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Meat and meat chain - Development of a new molecular diagnostic test

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“If you can dream it, you can do it.”

Walt Disney

To my parents, my brother and my grandmother.

To Jorge.

DECLARATION

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RESUMO

O objective do presente trabalho foi estudar o impacto do consumo da carne na saude humana e responder ao principal objective da tese: o desenvolvimento de um novo teste de diagnostico, baseado num Multiplex Real-Time PCR para detectar cinco bactérias: três patogénicos, *Salmonella* spp., *Campylobacter* spp. e *Listeria monocytogenes* e dois indicadores de higiene, *Escherichia coli* e *Staphylococcus* coagulase positiva em matrizes alimentares. Finalmente, foram analizados talhos, as suas caracteristicas e o impacto das boas práticas no risco de contaminação cruzada.

Para cumprimento do objetivo primordial, foi desenvolvida uma reflexão critica de forma a conhecer em profundidade as diferentes perspetivas evolutivas, históricas, religiosas e sociais do mercado da carne e a sua importância como alimento de prestigio, como fonte primordial de proteínas de elevado valor, energia e outros nutrientes exclusivos. Paralelamente, foi avaliado o nível de cumprimento das boas práticas em talhos da região Centro de Portugal e o nível de cumprimento desses mesmos estabelecimentos, relativamente à legislação comunitária em vigor.

Na Secção I, Capitulo I, foi analisada bibliografia científica que demonstra, inequivocamente, a importância da carne no processo de hominização, bem como a importância da cadeia da carne na perspetiva económica, social, ambiental e sob uma perspetiva histórica e religiosa. Concluiu-se que, uma redução no consumo *per capita*, face às ultimas projeções da FAO, seriam fundamentais para potenciar a redução das doenças não transmissíveis associadas ao consumo excessivo deste alimento, tais como diabetes tipo II, doenças cardiovasculares, sobrepeso, obesidade e cancro, entre outras, bem como as doenças zoonóticas de origem alimentar, enfoque principal deste trabalho. Conclui-se assim que é importante promover a redução do consumo desta fonte proteica; desenvolver programas de saúde pública que sensibilizem a comunidade para a necessidade de um bom manuseamento, armazenamento e confeção, como vias de redução de contaminação cruzada e promover uma monitorização mais eficaz da cadeia produtiva com consequente cumprimento da legislação em vigor, que poderá

significar um decréscimo de infeções humanas por agentes patogénicos de origem alimentar. Em conclusão, foi possível aferir que, o consumo de carne por si não é nocivo, nociva é a quantidade e a qualidade da carne atualmente consumida.

O desenvolvimento do novo método de diagnóstico que pretende simplificar a monitorização das diferentes etapas da cadeia de comercialização da carne, facilitando a deteção de bactérias patogénicas e simultaneamente reduzindo os custos associados ao diagnóstico laboratorial, foi descrito na Secção I, Capitulo II. Recorrendo à tecnologia de Multiplex PCR em Tempo Real, foi desenvolvido um novo ensaio de primeira linha que possibilita a deteção, sem enriquecimento prévio, de 10UFC/g de carne para *Salmonella* spp. e *Escherichia coli*, 10^3 UFC/g para *Campylobacter* spp., 10^6UFC/g para *Listeria monocytogenes* e 10^4UFC/g de carne para *Staphylococcus* coagulase positiva. Este teste tem uma especificidade absoluta para as bactérias supracitadasademonstrou elevada eficiência mesmo sem enriquecimento prévio para três tipos de matrizes alimentares, carne, queijo fresco e sumo de fruta e vegetais.

Na Secção I, Capítulo III, foi aferido, através de um questionário e de uma lista de verificação, o cumprimento das boas práticas em talhos da região Centro de Portugal, bem assim como o cumprimento de alguns requisitos legais, definidos pela legislação europeia, e subsequente transposição para a legislação nacional. Foram avaliados oitenta e oito operadores aferindo-se questões de conhecimento e prática e procedeu-se à inspeção visual de setenta e três estabelecimentos, com enfoque nas questões de higiene. Concluiu-se que, apesar de mais de 90% dos operadores terem tido formação nos últimos dois anos, o *score* calculado nas questões de conhecimento e prática, não ultrapassou os 68%, tendo, no caso da inspeção visual ficado pelos 64%. Foram registadas falhas graves de higiene, sendo que 86.3% dos operadores, ainda manipula carne e dinheiro sem higienização das mãos entre processos. Conclui-se portanto, do desenvolvimento deste trabalho, que é urgente que as autoridades intensifiquem as ações de sensibilização junto destes operadores económicos e que simultaneamente, seja feita uma formação efetiva aos mesmos, promovendo a

sua tomada de consciência face à sua importância na melhoria da saúde pública e subsequente redução das toxinfeções alimentares.

ABSTRACT

In the present work our commitment was to study the impact of meat consumption in human health and answering to the main objective of this thesis: to develop a new diagnostic test, based on a Multiplex Real-Time PCR, to detectfive bacteria: three pathogens, *Salmonella* spp, *Campylobacter* spp. and *Listeria monocytogenes* and two hygiene indicators, *Escherichia coli* and *Staphylococcus* coagulase positive in food matrices.Finally, to analyzebutcher shops, their characteristics and the impact of the manufacturing practices in cross-contamination risks.

To meet the primary goal, a critical reflection was developed in order to know in depth the different evolutionary, historical, religious and social perspectives of the meat market and its importance as a prestigious food, as a primary source of high protein value, energy, and other unique nutrients. In parallel, the level of compliance with good manufacturing practices and Community legislation was evaluated in butcher shops in the central region of Portugal.

In Section I, Chapter I, the scientific literature analyzed showed the unequivocal importance of meat in the process of human evolution, and the importance of the meat chain not only from an the economic, social and environmental perspective but also from a historical and religious perspective. It was concluded that a reduction in *per capita* consumption, compared to the latest projections of FAO, would be crucial to enhance the reduction of non-communicable diseases associated with excessive consumption of this food, such as diabetes, cardiovascular diseases, overweight, obesity and cancer, among others, as well as zoonotic food borne diseases, the main focus of this work. It is therefore concluded that it is important to promote the reduction of consumption of this protein source; develop public health programs to sensitize the community to the need for proper handling, storage and quilting, as cross-contamination reduction pathways and promote more effective monitoring of the production chain with consequent compliance with the legislation, which could mean a decrease in human infections of food borne pathogens. In conclusion, it was possible to

determine that the consumption of meat itself is not harmful, injurious is the quantity and quality of the currently consumed meat.

The development of this new diagnostic test aims to simplify the monitoring of the different stages of the meat supply chain, simplifying the detection of pathogenic bacteria and simultaneously reducing the costs associated with laboratory diagnosis, described in Section I, Chapter II. Using the multiplex Real-time PCR technology, a new first-line test has been developed which allows the detection without prior enrichment, 10 CFU / g of meat for *Salmonella* spp. and *Escherichia coli*, 10^3 CFU / g for *Campylobacter* spp., 10^6CFU / g for *Listeria monocytogenes* and 10^4CFU / g of meat for *Staphylococcus* coagulase positive. This test has an absolute specificity for all bacteria and demonstrated high efficiency even without prior enrichment for three types of food matrices, meat, cheese and fresh fruit and vegetable juice.

In Section I, Chapter III, via a questionnaire and a checklist, the level of compliance with good manufacturing practices in butcher shops of central Portugal, as well as the fulfillment of certain legal requirements by European legislation, and subsequent transposition into national law was assessed. Eighty-eight meat handlers for their knowledge and practiceand a visual inspection was performed at seventy-three establishments, focusing on hygiene issues. It was concluded that despite more than 90 % of the handlers having been trained in the last two years, the score calculated on issues of “knowledge” and “practice”, did not exceed 68 %, and in the case of visual inspection did not exceed 64 %. Serious hygiene failures were recorded, 86.3% of the meat handlers, manipulating meat and money at the same time, without proper hand hygiene. It is concluded, via the development of this work, that it is urgent that authorities intensify awareness-raising programs for these economic operators and that effective training is simultaneously given to them, promoting awareness of their importance in improving public health and subsequent reduction of food borne diseases.

Abbreviations

€ - Euro

AFLP - Amplified Fragment Length Polymorphism

bn – billion

CFIA - Canadian Food Inspection Agency

CFU – Colony-forming Unit

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

Ct - Cycle threshold

DAEC - Diffusely Adherent *E. coli*

DEC - Diarrhogenic *E. Coli*

DEFT - Direct Epifluorescent Filter Technique

DNA - Deoxyribonucleic acid

EAEC - Enteroadherent *E. coli*

EAggEC - Enteroaggregative *E. coli*

ECDC - European Centre for Disease Prevention and Control

EFSA - European Food Safety Authority

EHEC - Enterohaemorrhagic *E. coli*

EIEC – Enteroinvasive *E. coli*

ELFA - Enzyme-linked Fluorescent Assays

ELISA – Enzyme-linked Immunosorbent Assay

EN – European Norm

EPEC - Enteropathogenic *E. coli*

ETEC - Enterotoxigenic *E. coli*

EU - European Union

FAO - Food and Agriculture Organization of the United Nations

FISH - Fluorescence *in situ* Hybridization

g – gram

GBS - Guillain-Barré syndrome

GHPs - Good Hygienic Practices

GlcNAc – N - acetylglucosamine

GMPs - Good Manufacturing Practices

GPFI – Good Practice in Food Industry

h – hours

HACCP - Hazard Analysis and Critical Points

HEV - Hepatitis E virus

ISO - International Organization for Standardization

LCR –Ligase Chain Reaction

mCCDA - modified Cefperazone Charcoal Deoxycholate Agar

mL - millilitre

MLST - Multi Locus Sequence Typing

MLVA - Multi Locus Variable-number Tandem Repeat Analysis

MRSA - Methicillin-resistant *S.* coagulase positive

MurNAc – N – acetylmuramic acid

MVLST - Multi-virulence-locus Sequence Typing

NASBA - Nucleic Acid Sequence-based Amplification

NP – Norma Portuguesa

NTEC - Necrotocigenic *E. coli*

ºC – Celsius degrees

OIE - World Organization for Animal Health

PCR – Polymerase Chain Reaction

PET – Polyethylene Terephalate

PFGE - Pulsed-field Gel Electrophoresis

pH – power of Hydrogen

qRT-PCR - Real Time quantitative Reverse Transcription PCR

RAPD - randomly amplified polymorphic DNA

RFLP - restriction fragment length polymorphism

SE - *Salmonella enterica* serovars Enteritidi

SePEC - Septicemia Enteropathogenic *E. coli*

SNP - Single Nucleotide Polymorphism

ST - *Salmonella enterica* serovars Typhimurium

UNESCO - United Nations Educational, Scientific and Cultural Organization

UPEC - Uropathogenic *E. coli*

UK – United Kingdom

UN – United Nations

US – United States

VTEC – verotoxigenic *E. coli*

WHO - World Health Organization

WSH–Work Safety Hygiene

µm – micrometre

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SECTION I

INTRODUCTION

I. INTRODUCTION

1. The role of meat inhuman evolutionary perspective

The European legislation defines meat as the edible parts removed from the carcass of domestic ungulates including bovine, porcine, ovine and caprine animals as well as domestic solipeds, poultry, lagomorphs, wild game, farmed game, small and large wild game(EC 2004).

Some evolutionist theories referred a determinant role of meat on human evolution. During human evolution, four different meat consumption profiles are considered: the first could be characterized by an opportunist hunting; in the second, hunting had grown to a bigger scale; 2 to 3 million years later, in the third period, men started to domesticate animals and plants, which had begun 10,000 years ago; during the fourth and last period, several studies determined that meat contained compounds which could increase disease risk(Larsen 2003).

Meat is an important source of energy, metal ions, like iron or zinc, high-quality protein (composed by essential amino acids(Wu 2009)), vitamin A, vitamin B12, folic acid resource and heme compounds or other porphyrin iron rich compounds (Bothwell 1982, Biesalski 2005); it was extremely important in the development of *Homo sapiens* into an intelligent and social mammal(Leroy 2015); to humans transition from the forest to savanna and to the brain and intellectual development(Rotilio 2010, Leroy 2015), influencing a human erect posture (Abitbol 1995, Wang 2004), promoting a cranial-dental and bowel morphologic changes with increased energy needs, leading to an elevated quotient between brain and body size(Mann 2007). Gastrointestinal tract also had undergonesuccessive transformations, influenced by the diet and particularly by the meat consumption (Mann 2007).

Indeed, eating meat is a bio cultural act (Leroy 2015) with strong implications on human evolution.

2. Meat consumption, a critical issue on an healthy diet

A healthy diet is one of the most important factors that contributed to the human evolution(Leroy 2015) and to the human health preservation.Daily ingestion should include, different foods like grains, fruits, vegetables, legumes, fish and meat, which contribute with different nutrients for our body (e.g. carbohydrates, fats, proteins, vitamins and minerals) and should be eaten to achieve a healthy balanced diet(EUFIC 2009). Presently, scientific community discusses the role of diet in the etiology and prevention of chronic diseases and the relationship of the dietary patterns and nutrient intake to these diseases(NRC 1991, OMS/FAO 2003, EUFIC 2009).

According to World Health Organization (WHO), for an adult, a healthy diet should contain fruits, vegetables, legumes, nuts and whole grains, 5 portions of fruits and vegetables a day (OMS/FAO 2003), less than 10 % of total energy intake from free sugars (OMS/FAO 2003, WHO 2015a), less than 30 % of total energy intake from fats, particularly unsaturated fats (OMS/FAO 2003, FAO 2010, Hooper 2012) and less than 5g of salt per day (WHO 2012). The most recent WHO guidelines recommend a reduction of the red and fat meat and a restriction of the meat products consumption (WHO 2015b); Australian guidelines recommended as healthy diary protein intake, the consumption of 65 g of cooked lean red meat (beef, lamb, veal, pork, goat or kangaroo), or 80 g of cooked lean poultry (chicken or turkey), or 100 g of cooked fillet, or 120 g of eggs or 150 g of legumes/beans(AG 2015). UK, in accordance with WHO recommendations, encourages people to eat less red and processed meat, approximately 70 g/day (NHS 2016). USDA recommended ≈ 100g/day of meat(USDA 2015).

Human nutrition and eating habits are different around the World and are conditioned by political and natural events(Fara 2015). One of the most important recognized diets around the world is the Mediterranean diet (Byberg 2016). This has been recognized in 2010 as an intangible cultural patrimony of Humanity by United Nations Educational, Scientific and Cultural Organization (UNESCO), practiced also in many areas of Cyprus, Croatia, Spain, Greece, Italy, Morocco and Portugal (UNESCO 2012), and has been identified, classified and scientifically documented as the one that can guarantee longevity on good healthy by Ancel

Keys(Fara 2015). As he observed in 1950s in Naples, people had low incidence of coronary heart disease associated with the “good Mediterranean diet”; this diet generally includes high intake of vegetables, fruits and olive oil with low consumption of meat and dairy products(Keys 1995, Esposito 2016). Presently there are more than 100 different types of dietary theories.However, diets including high intake of meat and meat products have risks associated.

As will be mentioned further down, the increase of the meat and meat products consumption promoted healthy problems, especially metabolic disorders, coronary diseases and cancer(WHO 2015d). The problem is not the meat consumption but the unhealthy increase of the meat consumption.

3. Poor eating habits and health associated problems

Meat is an important energy and nutritional resource and was considered essential to optimal human growth and development(Higgs 2000). Despite this, radical dietary shifts in many developed and developing nations are supplanting traditional patterns, eating diets with high intake of meat products, refined carbohydrates and low intake of whole grains, fruits, and vegetables(Frank 2008, Rodrigues 2016). The emergence of several chronic diseases is associated with poor eating habits and sedentary lifestyle. Obesity, diabetes type II, cardiovascular diseases, cancer and oral pathologies are among the major health problems described by WHO and Food and Agriculture Organization of the United Nations (FAO) as caused by poor eating habits(OMS/FAO 2003).

3.1. Overweight and obesity

Overweight and obesity are one of the most reported problems, promoted by unhealthy eating habits(Cinelli 2016) caused by an imbalance between energy expenditure and energy intake(Anderson 2015, Kuroda 2016). Approximately 43 million individuals are obese, and childhood obesity predisposes the individuals to insulin resistance and diabetes type II, hypertension, hyperlipidemia, liver and kidney diseases and reproductive dysfunction in adults; over 90 % of obesity cases are idiopathic and less than 10 % are associated with genetic and hormonal causes (S. X. Xu 2016). Just in European Union (EU), in 2012, over half the

population was overweight or obese(Eurostat database 2015). Fat and carbohydrates intake (particularly refined products, like sugar), the food glycemic index and a poor fibers diet are the most important factors associated with obesity(OMS/FAO 2003, Te Morenga 2012, Parnet 2016). Metabolic alterations promoted by obesity enhance a lot of associated problems, like diabetes type II, cardiovascular diseases and an increased risk of cancer(Renehan 2010, Anderson 2015, Kuroda 2016).

3.2. Diabetes type II

The occurrence of diabetes type IIworldwide was estimated in 415millionsadults in 2015 and this value will increase to 642millions in 2040;just in 2015, 5.0 millions of peopledeath because of it (IFD 2015). This problem is associated with a poor diet with saturated fatty acids, like meat products and fat meatintake and rich in energy andpoor in fiber (OMS/FAO 2003).

3.3. Cardiovascular diseases

Cardiovascular diseases are an indirect problem caused by overweight, obesity, hypertension, dyslipidemia and diabetes type II(OMS/FAO 2003, Anderson 2015). A healthy diet with greater fiber intake(M. H. Xu 2015), vitamin D(Bi 2016), black and red berries, green and black teas and cocoa, omega-3 fatty acids(Auger 2016), low-salt(Suckling 2015) and reduced saturated fat, *trans* fat, red and processed meat, sweets and sugar-sweetened beverages is most important to promote a good heart and cardiovascular health(AHA 2015, de Oliveira Otto 2016). On the other hand, as humans have inherited a weak capacity to produce an important antioxidant and anti-inflammatory aminoacid, taurine (Wójcik 2010) from its precursors' methionine and cysteine(Chesney 1998).Thus it must be supplied through diet, particularly eating animal products, there exclusively source, as chicken and turkey dark meat(Pereira 2013).

3.4. Cancer

An increased risk of breast, colorectal, endometrium, kidney and esophagus cancer has been proven to be associated to poor diets. They were correlated to obesity in people(WHO 2002, Renehan 2010, WHO 2015b, Anderson 2015,

Muscaritoli 2016)particularly with less fiber intake like grains, fruit and vegetables and high levels of meat, meat products and saturated fats consumption, particularly in case of the colorectal cancer(WHO 2015d, Jun 2016, Brenner 2016, Graffouillère 2016, Kouris-Blazos 2016, Maćkowiak 2016, Tayyem 2016, Vlastarakos 2016, Li 2016).

3.5. Oral health problems

Nutrition health has an important role in oral health at young age, and is most important to the next teeth stages(A. Rugg-Gunn 1993, Türp 2016). Deficiencies of vitamin D and A and malnutrition, particularly protein energy malnutrition are associated to dental enamel hypoplasia and atrophy of saliva glands and increase teeth damage; furthermore, deficiency of vitamin C increases the risk of scurvy (OMS/FAO 2003). Moreover, food rich in refined sugars promote dental caries(A. H. Rugg-Gunn 1984, Stecksen-Blicks 1986, Burt 1988, Arean 1993, A. Rugg-Gunn 1993).

In synthesis, the control of health problems previously referred, according to our vision, needs more investments in healthyeducation, investment by national governments, political desire and social involvement (Muscaritoli 2016, Lindemann 2016). As will be described below, a poor diet with low intake of fruit, vegetables, and grains and rich in high fat, high-salt or high-sugar products, cause a lot of direct and indirect health problems. Despite of these general considerations, there is a clear political desire to counter such (Eisenbrand 2015), particularly the increase of meat consumption and associated problems. As Malgorzata Handzlik, draftsman of the opinion of the Committee on Internal Market and Consumer Protection, said in 2008 “(…) obesity and overweight represent challenges for contemporary society. They lead to many chronic conditions such as circulatory diseases, hypertension, type 2 diabetes, strokes and certain types of cancer. Combating obesity and overweight should be a priority for the Union’s health policy.”; Jens Holm, on behalf of the GUE/NGL Group, said in the same debate that, “(…) meat consumption is soaring across the world. If nothing is done, the UN Food and Agriculture Organization (FAO) warn that the already high consumption of meat will double by 2050. Meat contains saturated fats and contributes to obesity. Furthermore, the meat industry is seriously helping to

hasten climate change. The EU should phase out subsidies to the meat industry; yet, in the budget for 2007 alone, over 45 million euros were appropriated just for the marketing costs of the meat industry. It is counter-productive and, besides, a bizarre waste of taxpayers' money. The phasing out of these meat subsidies and a strategy for reduced meat consumption should be self-evident measures for better health in the EU."(European Parliament Debate 2008).According to FAO, an unhealthy increased of meat consumption is predicted between 2005/2007 – 2030, around 2.9% (FAO 2012).

There are some authors that identify the increased of the meat consumption as one of the problems that contribute to the overweight and obesity, and are correlated with all described health problems but, on the other hand, there are other studies, particularly in Europe and North America, that question and, in some cases, reported no association between intakes of unprocessed red meat and any cause of death, including cardiovascular disease(Kappeler 2013, Rohrmann 2013, Oostindjer 2014). This work was developed particularly to the meat market and with the special attention in biological hazards. Thus, the focus will be, not on poor eating habits, but on food safety hazards, correlated to food chain and particularly meat chain, and the risks for human health.

4. Food safety hazards: chemical and biological

Besides chronic diseases associated to the poor health diet, some chemical and biological hazardsare sources of concern after food consumption: microbes and chemical residues. There are considerable human health consequences with foodborne infections ranging from protracted illness to death and patients with impaired immunity are at greater risk(Datta 2012). The concept foodborne disease or foodborne illness is used to designate gastrointestinal complications that occur following recent consumption of a particular food or drink; WHO defines these as a "disease of infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water" (Loir 2003). Foodborne diseases are a major public health concern worldwide (Balaban 2000, Loir 2003, Eisenbrand 2015), millions of people suffer every year and the situation is quiet grave in developing nations creating social and economic strain (Dhama 2013), and it is a serious global public health problem.

Safe food is a basic human right; in 1996, the World Food Summit reaffirmed the inalienable right that each person across the globe has to access safe, adequate and nutritious food (Castell 2015). The widespread incidence of foodborne disease generates a substantial burden on society, although the full extent of the social and economic impact of such illnesses is difficult to measure, more precisely; the financial value for psychological costs, and indirect costs (Remond 2010).

Independently of the diet, food consumption involves contact with food safety hazards that have been categorized as biological, chemical, or physical agents in, or condition of, food with the potential to cause an adverse health effect(ISO 2005). According to *Codex Alimentarius*, a contaminant is “any substance not intentionally added to food or as a result of environmental contamination. The term does not include insect fragments, rodent hairs, and other extraneous matter”(CAC 2003). Unsafe food containing harmful bacteria, viruses, parasites or chemical substances, causes more than 200 diseases – ranging from diarrhea to cancers; according to WHO data, an estimated 600 million – almost 1 in 10 people in the world – fall ill after eating contaminated food and 420 000 die every year, resulting in the loss of 33 million healthy life per year(OMS 2015).

4.1. Chemical hazards

According to Canadian Food Inspection Agency (CFIA), chemical hazards occur when chemicals are present in food at levels that can be hazardous to humans. Chemical contaminations may occur through various pathways as, the environment, intentional use of chemicals, manufacturing processes and addition of food additives(CFIA 2014). In case of meat and meat products, the most important chemical hazards are environmental contaminants, food additives, processing-induced chemicals and veterinary drug residues(CFIA 2014). In the specific case of the veterinary drug residues, the use of substances having hormonal or thyreostatic action as well as β-agonists was banned in the EU, but sometimes forbidden drugs have been detected in feed through illegal administration to obtain an economical benefit(Reig 2007). These residues have been described as responsible for some forms of hormone-dependent cancers(EFSA 2007), intoxications by consumption of meat containing residues of

clembuterol (with a Portuguese case (Barbosa 2005)), allergic reactions or bacteria resistance (Butaye 2001)

In the United States (US), an evaluation that was made between 1998-2008, determined that 13.3 % deaths attributed to foodborne illnesses was promoted by chemical contaminants in terrestrial animals; particularly in poultry meat, 8.9 % of deaths were caused by these hazards(Painter 2013). According to European Food Safety Authority(EFSA) data, a total of 1,005,835 samples were reported by 28 Member States for analysis of substances and residues covered by the Directive 96/23/CE, 45 % were analyzed for substances having an anabolic effect and unauthorized substances and 61 % for veterinary drugs and contaminants. Of the 419,528 targeted samples, 0.31% were non-compliant(EFSA 2015b). Unfortunately, there are no European data related to the number of deaths caused by chemical contaminants in food.

4.2. Biological hazards

In addition to the chemical hazards, there are a lot of biological hazards on the meat chain. Scientific literature indicate that, in all food chain, there are 1415 species of infectious organisms known to be pathogenic to humans. Among them, 61 % are zoonotic and have economic impact in public health, with morbidity and mortality rates associated with all zoonotic agents (L. L. Taylor 2001a, E. Taylor 2001b). Biological hazards include parasites, virus and bacteria and occur when hazardous or pathogenic organisms are introduced in food and thus pose a food safety concern to consumers; it can be introduced in food from the environment or from inadequate sanitation practices and cross-contamination during all food chain(CFIA 2014). In 2014, a total of 5,251 foodborne outbreaks, were reported in the EU; overall, 45,665 human cases, 6,438 hospitalizations and 27 deaths were reported (EFSA and ECDC 2015c). The evidence supporting the link between human cases and food vehicles was strong in 592 outbreaks(EFSA and ECDC 2015c). The estimated number of unreported cases per annum is considerably high (Rohde 2015) and there is now an awareness of the member states towards reporting the real number of possible foodborne illness, thereby contributing to make collective consciousness of European Institutions.

4.2.1. Parasites

Besides the biological hazards, there are parasites, and the most commonly associated with foodborne illness are *Cryptosporidium parvum*, *Giardia duodenalis* or *G. intestinalis*, *Taenia* spp., *Toxoplasma gondii*, *Trichinella spiralis*, *Entamoeba histolytica* and *Entamoeba coli*(CFIA 2014). According to EFSA data, in EU, over 2.500 human cases of foodborne parasitic infections are reported each year; *Trichinella* spp., *Toxoplasma* spp. and *Giardia* spp. can be directly or indirectly transmitted between animals and humans through with contaminated food or water as source of infection(EFSA s.d.). According to Abdel-Hafaeez *et al* (2015), most important meat-borne parasites are *Toxoplasma gondii*, *Taenia saginata*, *Taenia solium* and *Trichinella spiralis*(Abdel-Hafeez 2015). *Toxoplasma* spp. was reported by the European member states on pigs, sheep, goats, hunted wild boar and hunted deer. *Toxoplasma*spp., just was detected in animals, 14 member states and 2 non-member states provided data, 9.7 % of positives in analyzed pigs, 3.9 % in cattle and 26.2 % in sheep and goats(EFSA and ECDC 2015c). In Portugal, 44 cases of toxoplasmosis in humans were detected in 2012, and until October 2013, have been detected 10 cases(Viegas 2014).

4.2.2. Viruses

Another biological food hazards are viruses, that are typically introduced into food either though poor personal hygiene practices, or via contaminated food ingredients.According to CFIA (2014), bacteriophage, enteric virus, hepatitis A virus, Norovirus, Norwalk virus and Rotavirus are the most commonly associated with food safety issues. In US, between 1998-2008, were reported 1,604 annual hospitalizations attributed to virus in poultry meat(Painter 2013). Among food-related illnesses in the US,66.6 % were transmitted by virus(Mead P.S. 1999). In Europe, in 2014, 20.4% of the foodborne outbreaks were caused by virus(EFSA and ECDC 2015c).Were reported 77 West Nile fever cases with 7 confirmed human deaths (EFSA and ECDC 2015c). According to data from 10 surveillance systems of the Foodborne Viruses in Europe Network, the Norovirus is found to be responsible for more than 85 % (n = 3.714) of all non-bacterial outbreaks of gastroenteritis reported from 1995 to 2000 in Europe(Lopman B.A. 2003). In Austria, in 2005, a total of 606 foodborne outbreaks were detected, affecting 1910

people and, Norovirus affected 22 of them(Much 2007).Globally, meat products (especially poultry) were responsible for 30 % of the outbreaks(Much 2007). Hepatitis E virus (HEV) is more commonly in tropical and subtropical countries but also was detected in Europe(Vasickova 2005).Authors consider that the close genetic relationship of the swine and human virus suggests that swine may be a reservoir of HEV and swine manure could be a source of HEV contamination of irrigation water or coastal waters with contamination of shellfish(Smith 2001).

4.2.2.1. Non-conventional transmissible agents

One of the mainly importantnon conventional transmissible agents for meat market are prions. Bovine spongiform encephalopathy (BSE) was epidemic between 1980 and 1996 and was one of the biggest food safety crisis in Europe with an inconsequent social bang(Prusiner 2004, C. W. Chen 2013, Court 2015).BSE is the most recently discovered zoonotic diseases and it belongs to a group of diseases called Tranmissible Spongiform Encephalopathies (TSEs) (EFSA 2014a). BSE cases were first reported in the United Kingdom in 1986(Lee 2013). Variant Creutzfeldt-Jakob disease (vCJD) that affects humans and is linked to the BSE epidemic in cattle,was first detected in 1996. Before the epidemic impact of this disease, with an epidemic peaked in 1992, it has been a decrease in the number of incidents (Lee 2013). These decrease was promoted by intensive surveillance and screening programs in the Western world (Lee 2013). However, BSE become manageable but not eradicated, as a consequence, more precautionary actions and approaches should be performed in order to control this disease (Saunders 2008).Nevertheless, a positive impact of these measures was observed in Europe, and particularly in Portugal. In 1996 were detected 31 animal cases, in 1999, 159 cases and in 2014 just one case (OIE 2016).

4.2.3. Bacteria

According to CFIA (2014), the principal bacteria associated with foodborne illnesses include *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium botulinum*, *Clostridium perfringens*, *Escherichia coli* 0157:H7, *Escherichia coli* 0104:H4, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, *Vibrio cholera*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Yersinia enterocolitica*

and *Cronobacter sakazakii*(CFIA 2014). The importance of different foodborne diseases varies between countries depending on foods consumed, food processing, preparation, handling, storage techniques employed, and sensitivity of the population(ICMSF 2002). Particularly in Europe, in 2014, Campylobacteriosis was the most commonly reported zoonosis, with 236,851 confirmed human cases and it continued to be high in poultry meat, followed by *Salmonella* spp. with a total of 88,715 cases reported;*Salmonella* spp. was the most often detected in meat and meat products. In case of *L. monocytogenes*2,161 human cases of foodborne disease were registered, in Europe in 2014(EFSA and ECDC 2015c).

5. The most important bacteria contaminantsin meat: *S.* coagulase positive, *E. coli*, *Salmonella* spp., *Campylobacter* spp. and *L. monocytogenes*

An effective monitoring of the whole food chain, and compliance of the current legislation, may significantly decrease human infections by foodborne pathogens. All of these have become a serious human health problem and it is of great importance to detect and trace the meat chain with rapid, sensitive, specific and economic diagnostic tools and change the techniques to replace or supplement classical microbiology tests to molecular tests (Fisher 2007). This could have a positive impact in the reduction of direct and indirect costs with the foodborne diseases described above. In addition to the human costs, it is also expected that the economic impact of these pathogens in industries decrease significantly. According to EFSA and WHO indicators, the most important foodborne pathogens in meat chain are *S*. coagulase positive, *E. coli*, *Salmonella* spp., *Campylobacter* spp. and *L. monocytogenes*.

5.1. Staphylococcus coagulase positive

The *Staphylococcus* genus is composed by 41 species and 24 sub-species (Jay 2001). *Staphylococcus* coagulase positive is the most commonly associated species with staphylococcal food poisoning, one of the most economically important foodborne diseases (Alarcón 2006).

S. coagulase positiveis Gram-positive, non-motile, commensal and opportunistic bacteria, responsible for several different infections such as invasive

disease which can be fatal. Diseases may vary from superficial to severe skin infections, toxic shock syndrome, pneumonia, infective endocarditis, and sepsis (Bergdoll 1981, Bohach 1990, Lowy 1998, McCormick 2001, Geissmann 2009, Que 2011).This bacterium is responsible for a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance (Loir 2003, Kadariya 2013).It is important to refer that there is currently a high occurrence of methicillin-resistant *S.* coagulase positive (MRSA) (Taubes 2008, Stach 2014). These organisms are highly tolerant to stressful environments and have the ability to survive at temperatures between 7.0ºC to 48.5ºC, pH between 4.2 to 9.3 and sodium chloride (NaCl) concentration up to 15 % (Chaibenjawong 2011). Due to its good adaptability, *S.* coagulase positive remains viable in meat handlers' hands, meat chain surfaces and food(Scott 1990, Kusumaningrum 2002, Loir 2003).

5.1.1. *Staphylococcus*coagulase positive diagnosis

Routine detection of *S.* coagulase positive in foodstuffs is usually carried out by traditional methods based on the use of selective media, for direct enumeration or the recovery isolates after enrichment in selective broth for 28-48h at 37.0ºC, after suspect colonies are confirmed by coagulase production. One of the reference methods is NP 2260:1986, another one is generically defined as Coagulase-positive staphylococci and the analytical reference method on European Regulation is EN/ISO 6888-1 or 2 (ISO 1999a, ISO 1999b). Commission Regulation (EC) No 2073/2005 regulates the presence of this foodborne bacterium and suggests that its presence in milk and dairy products or fishery products, for example, should lead to improvements in production hygiene and selection of raw materials (EC 2005).

It is important to refer the possibility of false negative results detection, especially in meat samples, because of the presence of protein/proteinase inhibitors, for example, enzymes with heme groups (Schrader 2012, Nagaraj 2013). For this reason, it is necessary to develop new generally and specific assays to determine the species, independently of the toxin that is being expressed at that moment. A polymerase chain reaction (PCR) method is a favorable alternative because is quick, low cost, easy to handle, sensitive and specific. This method would constitute a very valuable tool for microbiological

applications. Specific primers for PCR detection of *S.* coagulase positive have been directed for some genome regions as described in the following Table 1.

Table 1. Examples of genome regions used to identify *S.* coagulase positive with PCR diagnostic tests.

Gene	References
nuc gene	(Wilson 1991, Brakstad 1992)
enterotoxin genes	(Wilson 1991, Johnson 1991, Tsen 1992, Mäntynen 1997, Becker 1998)
tst gene	(Johnson 1991)
eta and *etb* genes	(Johnson 1991)
16S-23S rDNA spacer region	(Saruta 1997)
23S rDNA	(Straub 1999)

5.1.2. *Staphylococcus*coagulase positiveprevention

The presence of these bacteria is usually indicative of lack of hygiene in food production and/or food handling and there is no statistic prevalence data; for this reason, consumers need to be aware of potential food contamination at home. Cooking food thoroughly is important, but preventing contamination and cross-contamination and maintaining critical points are the most effective ways to prevent Staphylococcal foodborne disease (Kadariya 2013). In case of the meat production and meat chain, the implementation of Hazard Analysis and Critical Points (HACCP), Good Hygienic Practices (GHPs) and Good Manufacturing Practices (GMPs) and the maintenance of cold chain, good hygiene, adequate cleaning and disinfection of equipment's used in meat processing, are essential to prevent *S.* coagulase positive contamination (Lammerding 1997, Weese 2010, Hennekinne 2012, Bennett 2013, Syne 2013). Additionally, it is important to prevent handling in the case of skin, eye and/or nose infections because these are important sources of contamination.

5.2. *Escherichia coli*

Escherichia coli was firstly described in 1885 by Theodor Escherich, a pediatrician that isolated the bacteria from the feces of a child suffering of diarrhea (Escherich 1885). *E. coli* is a Gram-negative, catalase positive, non-spore-forming bacteria, typically with 1-5μm in length, and generally motile by peritrichous flagella. This bacterium has the ability to reduce nitrate to nitrite and produce acid and gas from de fermentation of D-glucose. There are commensal and pathogenic strains, which can be transmitted to human (Baylis 2011, C. Jensen 1893). Currently, the *Escherichia* genus is constituted by six species, E. *alberti*, *E.coli*, *E. coli inactive*, *E. fergunsonii*, *E. hermannii* and *E. vulneris*(Baylis 2011). This genus is part of the *Enterobacteriaceae* family, which is a great indicator of the level of hygiene in food and water industry, because their normal habitat is the gastrointestinal tract of mammals, birds and other potential contaminants.

During the last years, it was proposed different forms to distinguish pathogenic from non-pathogenic strains. One based on the clinical syndrome that *E. coli* causes in humans and animals. They are classically divided into strains :

i) with intestinal tropism, or diarrhogenic *E. coli* (DEC), like enterotoxigenic, enteropathogenic, enterohemorrhagic, verotoxigenic and entero-invasive ;

ii) with extraintestinal tropism, like uropathogenic *E. coli* (UPEC) and invasive [septicaemic *E. coli* (SePEC)](Mainil 2003a, Mainil 2003b, Mainil 2013)

Another identification method based on serotyping system was developed in 1940s by Kauffmann, and consists on the identification of surface antigens and divide these in somatic (O antigen), capsular (K antigen) and flagellar (H antigen) (Kauffmann 1947).

Concerning pathogenic strains to humans, especially to DEC, in 1970s three different classes were defined based on their specific properties, instead of the clinical syndrome they can produce:

i) enterotoxigenic *E. coli* (ETEC), which produce enterotoxins that cause hypersecretion of electrolytes and water by enterocytes;

ii) enteroinvasive *E. coli* (EIEC), which invade the human enterocytes;

iii) enteropathogenic *E. coli* (EPEC) which is recognized as pathogenic to humans, but whose virulence properties are still unknown at this point (WHO 1980, Mainil 2003b).

In the following years, more characteristics were discovered and more classes were defined based on the human disease, namely, verotoxigenic (VTEC), enterohaemorrhagic (EHEC), enteroadherent or enteroaggregative (EAEC or EAggEC), diffusely adherent (DAEC), necrotocigenic (NTEC), UPEC and SePEC (Nataro 1998, De Rycke 1999, Dho-Moulin 1999, Nagy 1999, Kaper 2004, Baylis 2011).

E. coli is an indicator of the level of hygiene in food industry, however total absence is not expected. Depending on the class and the strain characteristics, *E. coli* presents different infection mechanisms and consequently, different symptoms. Generically, all of the strains, VTEC, EPEC, EIEC, ETEC and DAEC cause watery diarrhea, some with blood and mucus, like EIEC for example. Others, like EPEC, cause an inflammatory response together with other typical symptoms. Specifically in case of EAggEC, patients usually present abdominal pain (Baylis 2011).

5.2.1. *Escherichia coli* diagnostic

Classical culture methods have a quantification limit of 4 CFU/mL for liquid food and 40 CFU/g for solid food (Jasson 2010). Based on European legislation, *E. coli* is used as fecal contamination indicator or as an indicator for hygiene levels, namely in meat and meat products, cheese, bivalves and other foodstuffs, and its presence is evaluated with the gold standard method, ISO 16649 (CEC 2005). Other diagnostic tests based in different technologies, to evaluate *E. coli* presence in foodstuff have been described, namely:

i) Flow cytometry, measures the optical characteristics of cells; the fluorescent dyes can be used to probe the viability and metabolic state of pathogens. This method is often used in combination of live-dead staining or in

combination with viability staining for metabolic activity indication(Tanaka 2000, Gunasekera 2003, S. D. Flint 2006, S. W. Flint 2007);

ii) Direct Epifluorescent Filter Technique (DEFT), microscopic cell counting method where samples are concentrated and collected on a membrane, where they are stained with fluorescent dies. Epifluorescence is used to analyze the filter surface. This detection can be automated if the microscope is linked to an image analyzing system (Jasson 2010);

iii) Real Time quantitative Reverse Transcription PCR (qRT-PCR), an alternative detection method based on the ability of two single-stranded DNA molecules in vitro to hybridize by specific base pairing. Standard curves can be established to correlate the cycle threshold (Ct) values of the PCR to genomic copies in DNA extract (Maurer 2006). This technique allows quantification of genomic copies based on the relation between genomic copies and the number of cells present in the sample. The quantification limit is approximately 10^3-10^4 cells per gram, which is too high for practical applications because most samples collected from food chain are contaminated with low numbers of pathogens (usually less than 100CFU/g) (Jasson 2010).

5.2.2. *Escherichia coli* prevention

According to EFSA, consumers can reduce the hazards of falling ill from potentially contaminated food by following good hand hygiene and food handling practices. These include refrigerating foods promptly, regularly washing hands and surfaces, separating raw meats from other foods and cooking food at the right temperature(EFSA 2015a). Being an indicator of the level of hygiene in food industry, there are no reliable data as to the prevalence.

5.3. *Salmonella* spp.

Salmonella spp. has been documented as a source of illness since the late 1800s (Fatica 2011). *Salmonella* is Gram-negative, gammaproteobacteria, short plump shaped rods, non-spore-forming, non-capsulated, aerobic and facultative anaerobic organism and classified under the family *Enterobacteriaceae* that are often pathogenic to humans causing salmonellosis (McQuiston 2008, NCEZID

2011). *Salmonella* spp. grow under temperature range between 6.0ºC to 46.0ºC, with 37.0ºC as optimum temperature, pH range between 4.1 – 9.0, with an optimum pH between 6.5 to 7.5(Delhalle 2009, NCEZID 2011).

In 1987, it was proposed to divide the genus in two species, based on DNA similarity (Minor 1987). This division in two species was maintained in what is considered “the contemporary classification”, shown in Figure 1(Grimont 2007).The typical format for a serotype formula is Subspecies [space] O antigens [colon] Phase 1 H antigen [colon] Phase 2 H antigen (Popoff 1997, NCEZID 2011). The Kauffmann-White scheme is a document where are listed all the simplified antigenic formulae of *Salmonella* serovars(Popoff 1997) and this list is defined and maintained, annually by the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France.

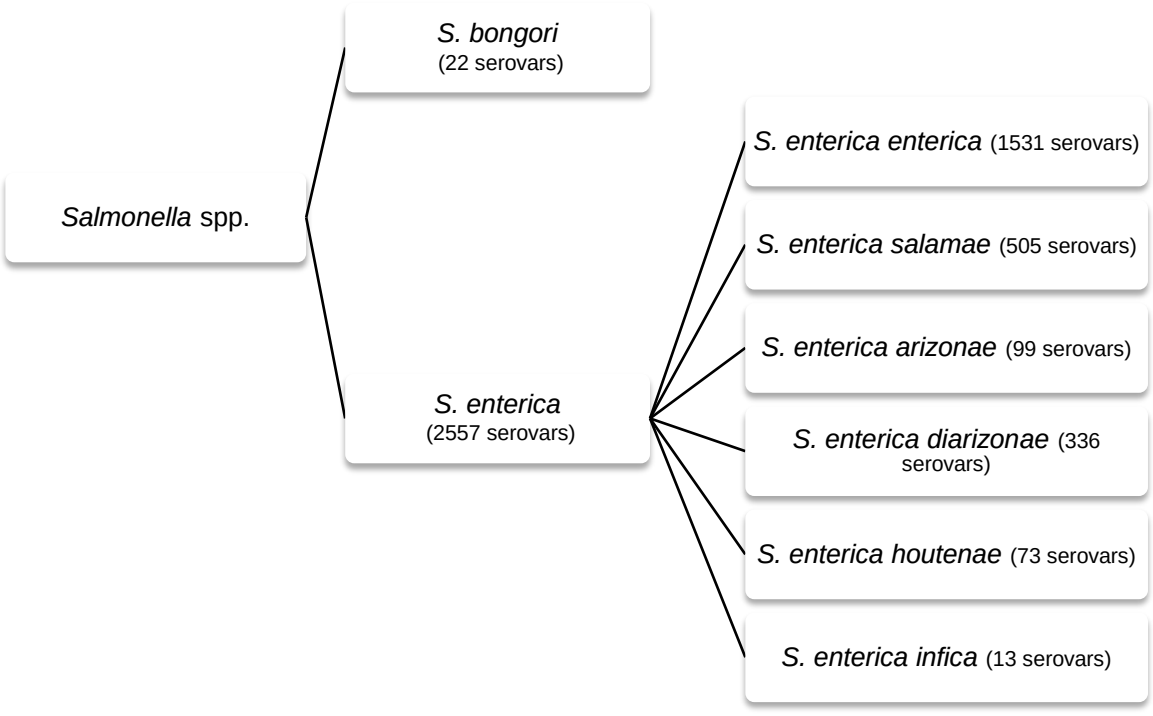

Figure 1. The contemporary classification to *Salmonella* spp(based on Grimont 2007).

Salmonella spp. typically causes gastroenteritis and typhoid fever in humans, but non-typhoid strains commonly cause bacteremia and meningitis (Sirinavin 1999). *Salmonella* spp. is recognized worldwide as a major human foodborne pathogen (CDC 2008) and its presence in foodstuffs represents an internationally accepted human health concern (Carraco 2012). The human infection is characterized by no inflammatory or secretory diarrhea of watery stools without the presence of white blood cells in feces.

5.3.1. *Salmonella* spp. diagnostic

For more than 80 years, subtyping of *Salmonella enterica* has been routinely performed by serotyping, a method based on agglutination reactions between surface antigens and specific antibodies (P. B. Wattiau 2011). Currently, EU defined an analytical reference method for *Salmonella* detection, EN/ISO 6579 for a large range of foodstuffs, as minced meat and meat preparations, milk and milk products, ice cream, eggs, ready-to-eat food, live bivalve mollusks and live echinoderms, tunicates and gastropods, fruit and vegetables and others.

In 1990s, pulsed-field gel electrophoresis (PFGE) was adapted to *Salmonella* spp. diagnostic(Olsen 1994, Weide-Btjes 1998, Garaizar 2000, Murase 2004) and was long considered the gold standard for *Salmonella* molecular subtyping (P. B. Wattiau 2011). Recently, the scientific community has tried to develop new methods, based on DNA analyses, but it is not clear which is the optimal detection method, being PFGE still considered the gold standards for subtyping *Salmonella* strains. Currently it is possible typing *Salmonella* using two types of methods, phenotypic and molecular methods.

The phenotypic method is based on serotyping procedure, and there are described two different targets, one is based on agglutination of bacteria with specific sera to identify variants of the somatic and flagellar antigens (Grimont 2007, McQuiston 2008). The main disadvantages are the need of 150 specific antisera and trained personal. Another method is based on SuperEpoxy microarray slides spotted with antibodies and fitting the Kauffmann-White scheme. This method detects between 80 % and 90 % of *Salmonella* species collected in Canada (Cai 2005). The antibody-antigen reaction is conducted on a microvolume

scale on slides following fluorescent labeling of the investigated *Salmonella* strain. The main advantages are the reduced time analysis, standardized agglutination detection and simultaneous detection of O and H antigens (P. B. Wattiau 2011).

The molecular method is based on molecular typing techniques. Some examples are described below:

i) PFGE – this is used for fingerprinting strains in outbreak situations and is relatively inexpensive but time-consuming and labor-intensive and does not display equal sensitive for different serovars (Kerouanton 2007);

ii) molecular serotyping based on lipopolysaccharide and flagellar structural genes – this is used to identifying *Salmonella enterica* based on the genes that code for the somatic O and H antigens. For this purpose there were developed different assays over 10 years (Echeita 2002, S. M. Herrera-León 2004, S. R. Herrera-León 2007, Fitzgerald 2007). The main advantages are the requirement of reduced technical expertise and the rapid results delivery;

iii) molecular serotyping based on genomic markers – There are some assays based on molecular methods, based on specific targets to detect and differentiate serovars of *Salmonella* spp. (Andreoli 2004, P. V. Wattiau 2008), like a molecular serotyping system based on the multiplex ligase chain reaction (LCR) assay, analyzed on a low-density DNA microarray platform, has been made commercially available as Check&Trace *Salmonella* by Check-Points. Another method is based on the PCR amplification of 12 genetic *loci* specific for some serovars (Kim 2006). This test allows the identification of 28 most common, clinically relevant *S. enterica* subspecie *enterica* serovars, identified in the US. Actually, this method was further developed and was created a “PCR on a chip” where PCR products amplified *in situ* and detected with an universal fluorescent hybridization probe (Mertes 2010);

iv) molecular subtyping by microarray technology – several microarray setups were used to screen the genome of *Salmonella* species. It was developed an assay that simultaneously determines the molecular characterization and typing of *Salmonella enterica* subsp. *enterica* isolates using flagellar and somatic antigen

encoding genes, virulence genes located within or outside the pathogenicity islands, phage-associated genes, and antibiotic resistance determinants (Malorny 2007). This microarray contains 109 35-to 40-mer oligonucleotide probes (P. B. Wattiau 2011).

The World Organization for Animal Health (OIE) continues to review current and new methods for identification and surveillance of *Salmonella* spp. in foodstuffs. Nevertheless, PFGE is still used as the reference method.

5.3.2. *Salmonella* spp. prevalence

A consumer has a 50:50 % chance of carrying home live *Salmonella* spp., with foodstuffs provided from the supermarkets. It was reported 40.3 % of *Salmonella* positive samples of ground meat from swine, 46 % from cattle and 56.3 % from processed poultry products (Teuber 1999). *Salmonella* strains from food animals are transmitted to humans via insufficiently cooked meat, eggs, and milk (D'Aoust 1997). Human salmonellosis is mainly caused by the consumption of raw or partially cooked eggs contaminated with *Salmonella enterica* serovars Enteritidis (SE) or Typhimurium (ST), which may also be transmitted by contaminated poultry meat; SE is the most reported serovar, responsible for foodborne salmonellosis around the world. The global prevalence of *Salmonella* food poisoning has gone up significantly since 2001 (Hendriksen 2011), and this has caused a significant financial burden on the health care system (Korsgaard 2009).

Salmonella spp. is a zoonotic agent tightly regulated and since 2003, the criteria for the presence/absence of *Salmonella* in breeding flocks of *Gallus gallus*, laying hens of *Gallus gallus*, broilers of *Gallus gallus* and breeding and fattening turkeys have been successively updated. In order to protect the human health, the EU settled clear targets to reduce the presence of specific serovars, applying the Commission Regulation (EC) No. 2160/2003. The definition of these serovars was progressive, started with *S.* Enteritidis and *S.* Typhimurium during first three years, and to breeding flocks of *Gallus gallus* were defined *S.* Hadar, *S.* Infantis and *S.* Virchow. These five serovars were the most reported in human salmonellosis(Messens 2013). Based on an EFSA review, before the end of the

transitional period, a review of the EU target should be carried out and other serovars with public health impact should be considered. The *Salmonella* serovars with public heath significance were determined by EFSA, based on Annex III to Commission Regulation (EC) No. 2160/2003 (Messens 2013). According EFSA and ECDC, in 2014, a total of 88,715 confirmed salmonellosis cases were reported by EU member states; this represents an increase of the 15.3 % compared with 2013. Sixty-five fatal cases were reported by eleven member states (EFSA and ECDC 2015c). The two most commonly reported *Salmonella* serovars in 2014 were *S*. Enteritidis and *S.* Typhimurium, representing 44.4% and 17.4%, respectively,in human cases(EFSA and ECDC 2015c). In animals, the EU-level prevalence of *Salmonella* target serovar-positive poultry flocks was very low (<1 %) for breeding flocks of *Gallus gallus*, for laying hen flocks, broiler flocks, as well as for flocks of breeding and of fattening turkeys.

5.4. *Campylobacter* spp.

The first report of *Campylobacter* spp. it is believed that was made in 1886 by Theodore Escherich that called "*Cholera infantum*" (Snelling 2005, King 2008, Vandamme 2010, Silva J. 2011, Epps 2013)and was identified in 1906 by John McFadyean and Stewart Stockman (Skirrow 2006).

Campylobacter species comprising Gram-negative, curved or spiral-shaped (0.2– 0.8 μm), microaerophilic bacteria (Bolton 2015), requiring an oxygen concentration of 3 %±15% and a carbon dioxide concentration of 3 ±5% and normally spiral-shaped, have been reported to change into coccoid forms on exposure to atmospheric oxygen levels or other stressors, with a polar flagellum at one or both ends of the cell. The temperature range for multiplication of the thermophilic *Campylobacter* species *C. jejuni* and *C. coli* are 34.0ºC±44.0°C, with an optimal temperature of 42.0°C (van Vliet 2001).Some authors suggest that *Campylobacter* genus comprises 20 species and subspecies (Fernández 2008) and others suggested that there are 17 species and 6 subspecies (WHO 2011). Among the genus, the most common in foodborne diseases are*C. jejuni* subspecie *jejuni* and *C. coli*, particularly in Europe and North-America (Rokosz 2014). Other species as *C. lary* and *C. upsaliensis* were also isolated in patients with gastrointestinal diseases, but these are less frequent (WHO 2011).

The infections caused by enteric *Campylobacter* spp. not only promote fever, vomiting and headaches, gastroenteritis, or inflammation of the intestines but, can also promote serious squeal such as Guillain-Barré syndrome (GBS) and reactive arthritis (Zia 2003, WHO 2011). Depending on the characteristics of the country and the target population, the symptoms can be different, as mentioned inTable 2.

Table 2.*Campylobacter* spp. infection in human, symptoms based on population target(based on Ketley 1997, Wooldridge 1997).

Country characteristics	Target population	Symptoms	Time of year
Industrialized	Young adults Young children	Inflammatory diarrhea, severe cramping	Seasonal
Developing	Asymptomatic carriage	Milder clinical symptoms of watery	Not seasonal
	Young children	Non-inflammatory diarrhea	

It is expected that differences observed between populations of different countries remain due to the acquisition of immunity in developed countries population and not due to the existence of geographical variation in strain types. This is confirmed by the observation that travelers infected by *Campylobacter* spp. had similar symptoms according to their country of origin and not the country they had visit (Ketley 1997).

5.4.1. *Campylobacter* spp. diagnostic

The internationally recognized methods for the detection and enumeration of *Campylobacter* species in all types of food samples are respectively, ISO 10272-1:2006 and ISO 10272-2:2006. The mathematical lower limit of enumeration using this method is 1 or 2 CFU/mL of liquid samples, or 10 or 20 CFU/g to other food products (Public Health England 2014). To perform this

method, is used routinely a modified Cefperazone Charcoal Deoxycholate Agar (mCCDA) and on this medium, typically, *Campylobacter* species form greyish, flat and moist colonies, often with a metallic sheen, and with tendency to spread. As previously described, the most common species are *C. jejuni* and *C. coli*, but this method does not distinguish the species, and in case of doubtful results, samples are sent to reference laboratories to complete the identification (Public Health England 2014). Reference methods are time-consuming, cumbersome and not amenable to automation for screening of large sample numbers (Josefsen 2015). It is therefore important to developed new methodologies based on molecular methods, but currently, there are just a few descriptions of molecular techniques.

5.4.2. *Campylobacter* spp. prevalence

According to EFSA and ECDC data, *Campylobacter*continued to be the most commonly reported gastrointestinal bacterial pathogen in humans in UE and is responsible for 236,851 cases of infections (EFSA and ECDC 2015c). In terms of economic impact, infections by *Campylobacter* spp. costing approximately €2.9bn per year (EFSA 2012). Considering the high number of human campylobacteriosis cases, the severity in terms of reported case fatality was low (0.01%)(EFSA and ECDC 2015c). Interestingly, the apparent increase in the proportion of Campylobacter-positive broiler meat samples from 2012 to 2013 (31.4 % positive samples of fresh broiler meat) was mainly due to the inclusion of findings in Croatia, who only reported data for the first time in 2013. According to 2010 data, the poultry reservoir is responsible for an estimated 80 % of human infections by *Campylobacter* spp. (EFSA 2010). Another important result is that*Campylobacter* spp. was also detected in turkey meat at moderate levels and in other food at low to very low levels, according to 2013 data (EFSA 2015b). *C. jejuni* was responsible by 90 % of the cases(Bolton 2015). In 2014, 38.4 % of the 6,703 samples of fresh broiler meat were found to be *Campylobacter* positive, as described in 2013(EFSA and ECDC 2015c).

5.5. Listeria monocytogenes

L. monocytogenes was first described by E.G.D. Murray in 1926 (Cossart 2007) and belongs to *Listeria* genus. This genus is composed by six species, *L.*

monocytogenes, *L. seeligeri*, *L. ivanovvi*, *L. welshimeri*, *L. grayi* and *L. innocua*, based on the rRNA 16S homology, chemotaxis properties and enzymatic analysis (Rebuffo 2005). For humans, just *L. monocytogenes* is pathogenic (Cossart 2007).*L. monocytogenes* is an environmental ubiquitous foodborne pathogen, facultative intracellular parasitic bacterium (Esteban 2004, Hasegawa 2014), Gram-positive, lives in pH range between 5.0 to 9.6, is extremely tolerant to alkaline stress, dehydration, cold and osmotic stress(Sue 2004, Sauders 2007, Severino 2007, Raengpradub 2008) and it is present in soil and vegetation, sewage, water, animal feeds and food processing environments (Liu 2003, IFT 2004). *L. monocytogenes*multiplies at refrigeration temperatures, in opposition to many other foodborne pathogens, which contributes to the difficulty of its control. This species includes thirteen serovars, which are distinguishable by surface carbohydrate and flagellar antigens (Orndorff 2006, V. S.-C.-S. López 2006).

L. monocytogenes may have different virulence levels; some strains cause virulence and death while other show low virulence levels or are non-virulent (Liu 2003, McLauchlin 2004, V. O.-S. López 2008). Different ways of infection have been described, the most common is ingestion, through consumption of contaminated food, namely meat or fish poorly cooked or eaten raw, milk and lactic products unpasteurized, raw vegetables and other examples; other infection mechanisms include direct contact animal-human, human-human, inhalation and venereal transmission (WHO 2004). In case of newborns, the infection is vertical and *L. monocytogenes* is transmitted through the placenta or at a birth (WHO 2004).

The infection by *L. monocytogenes* results in a pathology called listeriosis, although it is rare it can be quite severe and those more prone to contract the illness are pregnant woman, new born, elderly people and immunocompromised patients. Three main invasive forms are described: septicemia, neurolisteriosis and maternal-neonatal infection (Leclercq 2014). In pregnant women, the infection can be dangerous for the fetus, it can cause sepsis, granulomatosis, respiratory disease and meningitis and the pregnant women just have flu-like symptoms (WHO 2004). In healthy people, *L. monocytogenes* can cause gastroenteritis, nausea, diarrhea, headaches, abdominal pain and myalgia (WHO 2004,

Swaminathan 2007). In addition, some people have papular or pustular skin rash (WHO 2004).

5.5.1. *Listeria monocytogenes* diagnostic

Similar to other foodborne pathogens, *L. monocytogenes* has an international reference method, ISO 11290-1 (ISO, 1996, 2004) regulated by the EU(ISO 1996). Non-reference methods are highly diverse; a non-exhaustive description will be presented with some of the molecular methods being briefly described. Traditionally, for detection of *Listeria monocytogenes* in food samples, an enrichment step between 24 hours to 72 hours is essential. This increases the number of bacteria necessary for detection by classical methods. Because of this method limitations and the current development of diagnostic detection methods, alternative methodologies have been proposed, namely molecular methods like PCR which enable the determination of the serotype (Table 3), or to confirm traditional methods, for example using the *prfA* specific primers (D'Agostino 2004). Other primer pairs described are specific to *hlyA* gene (Blais 1997, Lehner 1999, Lehner 1999, Hudson 2001, Lunge 2002, Hough 2002, Lübeck 2003, Rodríguez-Lázaro 2004), *iap* gene (Cocolin 2002, Schmid 2003)*inlB* gene (Ingianni 2001, Lunge 2002, Jung 2003) or 16S rRNA (Schmid 2003) but, according to some authors, the most commonly used has been*hlyA* gene (Aznar 2002). Other molecular methods and nucleic acid-based methods have emerged in recent years, like Multi Locus Sequence Typing (MLST) (Hasse 2014), Multi Locus Variable-number tandem repeat Analysis (MLVA) (Miya 2008), Multi-Virulence-Locus Sequence Typing (MVLST)(Zhang 2004, Y. Z. Chen 2005, Y. Z. Chen 2007), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) (Sesto 2014) and Single Nucleotide Polymorphism (SNP) (Ward 2008, Lomonaco 2011), Nucleic Acid Sequence-Based Amplification (NASBA) and DNA microarrays. There are other methods that can identify the subtype of the pathogen present in a sample, for example, Restriction Fragment Length Polymorphism (RFLP), PFGE (Okwumabua 2005), Amplified Fragment Length Polymorphism (AFLP), Randomly Amplified Polymorphic DNA (RAPD) or Fluorescence *In Situ* Hybridization (FISH) (Churchill 2006).

Table 3. Multiplex PCR primers, used for the determination of the serotype of *Listeria monocytogenes*(Doumith 2004).

Genetic target	Concentration (µM)	Primer sequence	Amplicon size (bp)
Imo0737	0,13	Fw AGGGCTTAAGGACTTACCC Rw ACGATTTCTGCTTGCCATTC	691
Imo 1118	0,3	Fw AGGGGTCTTAAATCCTGGAA Rw CGGCTTGTTCGGCTACTTA	906
ORF2819	0,13	Fw AGCAAAATGCCAAAACTCGT Rw CATCACTAAAGCCCATTG	471
ORF2110	0,3	Fw AGTGGACAATTGATTGGTGAA Rw CATCCATCCCTTACTTTGGAC	597

Besides these methods, there are immunoassay-based methods that allow the identification of the *L. monocytogenes*, like enzyme-linked immunosorbent assay (ELISA), agglutination and immunoprecipitation assays or enzyme-linked fluorescent assays (ELFA).

5.5.2. *Listeria monocytogenes* prevalence

Nyfeldt reported the first case of human infection by *Listeria monocytogenes* in 1929 (Nyfeldt 1929). In European member states, 2,161cases were reported only in 2014, with a case fatality rate of 15 % (EFSA and ECDC 2015c). The number of listeriosis cases in EU, increased a 30 %, compared with 2013 (EFSA and ECDC 2015c). In EU, surveillance focuses only on cases of severe infections and which represents only a small proportion of all *Listeria* infections in humans. This bacterium was mostly found in ready-to-eat fish and meat products; based on scientific reports of the past decade, compared with other foodborne pathogens, *L. monocytogenes* causes fewer infections than non-typhoidal *Salmonella*, *Salmonella* Typhi (Crump 2004), or *Toxoplasma gondii* related to congenital cases (P. M. Torgerson 2013). *L. monocytogenes* causes around 18,000 cases (P. K. Torgerson 2010), and also caused fewer deaths than did *S.* Typhior non-typhoidal *Salmonella*, with around 155,000 deaths (Majowicz 2010). Ninety percent of listeriosis cases in human are contracted through the consumption of contaminated food. (Scallan 2011). Although, as described above,

there are a few invasive cases, the mortality and morbidity rates for the invasive form of the pathology ranges between 20 % to 40 %, with a hospitalization rate over 92 % (Farber 1991, Ward 2008, Allerberger 2010, Yin 2015).Three of thirteen serotypes of *L. monocytogenes* (1/2a, 1/2b and 4b) are responsible for more than 95 % of clinical cases (Yin 2015). Specifically, the 4b serotype, is responsible for 33 % to 50 % of sporadic human cases worldwide (Den Bakker 2010). It should be noted that from 1 % to 10 % of the population are fecal carriers of *L. monocytogenes*(Hernandez-Milian 2014). Until October 2013, in Portugal 13 acute hospital admissions were identified, in 2012 were reported 35 infectious intestinal diseases caused by *L. monocytogenes* and 24 cases in 2011(Viegas 2014). In 2014, EU member states reported *Listeria* in cattle, sheep, goats, pigs and solipeds but *Listeria* was also detected in broilers, cats, dogs, hunted wild boar, foxes, and other wild and zoo animals (EFSA and ECDC 2015c).

6. Control measures in meat chain

All of these five bacteria have as similar characteristic, the adaptability of extreme conditions and subsequently survivability. All of these have the great capacity to live in meat and meat products and in all steps of meat production chain (Figure 2). The control measures in meat chain should be taken based on HACCP principles, GMP and GHP. Safe handling of food and good kitchen hygiene can prevent or reduce the hazards posed by contaminated foodstuffs.

More than the control of meat chain in pre and post-harvest steps, is important to control the growth of microorganisms, promoted by extrinsic and intrinsic factors. The most important extrinsic factors are: temperature, humidity rate and oxygen concentration; the most important intrinsic factors are: pH, water activity, nutrient content, structure of food items(The Open University UK 2016).

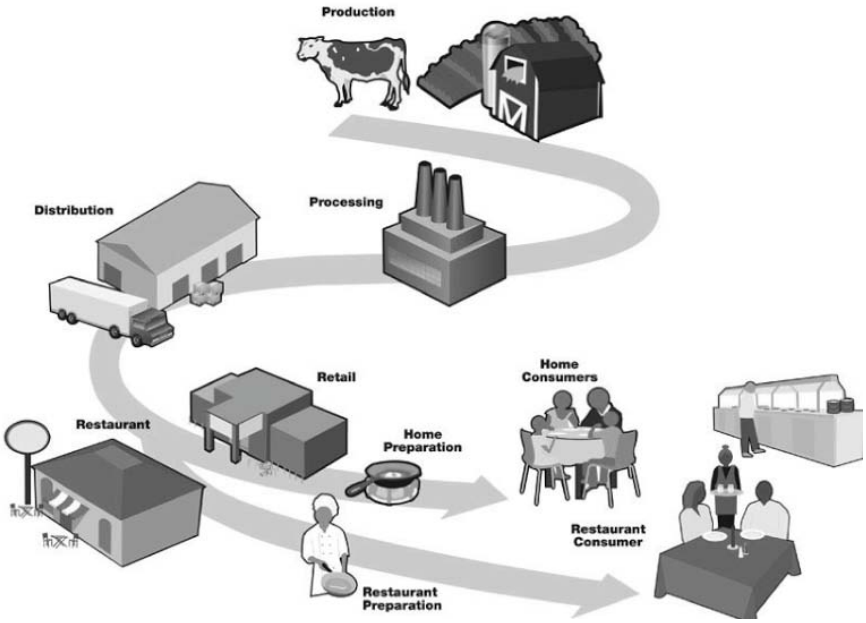

Figure 2. Steps of food production chain (adapted CDC 2011).

Finally, the last steps of this chain are developed at home and is crucial for consumers to be aware of the hazards and minimize these in home environment, with temperature treatments, storage and cooking procedures, to control the cross-contamination.

SECTION II

AIMS OF THE THESIS

II. AIMS OF THE THESIS

"Food safety: a right or a privilege?" In 2014, FAO/WHO brought together a multi-disciplinary panel to deliberate on why food safety is an essential element of food and nutrition security. According to FAO, food security is built upon four pillars including food availability, access, utilization and stability. Within this framework, food safety, food security and nutrition are inextricably linked, however the importance of food safety is often overlooked(FAO 2014). Where food is unsafe, individuals who are already vulnerable to food insecure are exposed to chemical, biological and other hazards that can pose serious acute and chronic health risks (FAO 2014). Just in EU, a total of 5,251 foodborne outbreaks were reported in 2014 by EFSA and ECDC, 45,665 human cases, 6,438 hospitalizations and 27 deaths(EFSA and ECDC 2015c) and around the world, approximately 2 million people per year die from diarrhea diseases from contaminated food and water, most of them children(FAO 2014).

The aim of this work was contribute to the control of bacterial contamination at the last steps of the meat chain. In this attempt, we develop a new diagnostic test, based on the PCR amplification; a Multiplex Real-time PCR that detects, on the same reaction, three pathogens (*Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes*) and two hygiene indicators in meat chain (*Escherichia coli* and *Staphylococcus* coagulase positive).

Specific aims of the studies included in the thesis were:

a. Study different perspectives of the meat chain, particularly health, economic, environmental and social problems, and mainly an historical dimension;
b. Design and validate a new Multiplex real-time PCR, to detect, at the same time, *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes*, *E. coli* and *Staphylococcus* coagulase positive;
c. Analyze one of the last steps of the meat chain, the butcher shops. Evaluate the impact of the compliance of the good practices by butchers, in Portugal and the compliance of the European regulation.

The last step of this study was conducted in butcher shops of the Center Portugal, and the development of the experimental work and respective results are present in the following chapters under the form of research paper.

SECTION III

RESULTS

CHAPTER I. Meat: prestigious or indecorous?

CHAPTER I. Meat: prestigious or indecorous?

Authors: Ana Santos, Eduarda Gomes Neves, José M. Correia da Costa

Abstract: It is important to take an overall view, of the meat market. It is more than food consumption, is defined based on historical dimension, this is a bio cultural activity with strongly impact in the human development process. The consumption of meat and meat products has healthy, economic and social impact. Furthermore, meat is responsible forhigh incidence of metabolic and chronic diseases including diabetes type II or cardiovascular diseases, cancer and isone of the biggest source of infection for food borne, responsible for bacterial, chemical, parasitic and viral outbreaks. Presentlyit is determinantto analyze meat as a prestigious and indecorous food. The problem is not the meat consumption but the quantity, the quality and the safety of meat consumed.

Meat: prestigious or indecorous?

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Abstract

Meat has been a key component of the human diet since the earliest times of humanity. However, consumption of meat is controversial because it favors high incidence of metabolic and chronic diseases including diabetes type II or cardiovascular diseases, cancer, and food borne diseases. Does the human diet require meat? In our view, meat is a good and prestigious food fundamental to a well-balanced diet (100 gram/day/person) is respected. The notice of the Swiss physician Paracelsus (b.1493-d.1541) "*sola dosis facit venenum*" seems appropriate here.

Key words:meat consumption; human health; chemical residues; foodborne.

1. Introduction

Food supply has played a decisive role in the process of humanization. Consensually, worldwide, and in all languages, we assume: “we are what we eat”. In particular, consumption of meat is part of our evolutionary heritage (Smil, 2002); it is consensual the acceptance of it’s role on the definition of the contours of elegance and wit of modern man, basically due to the biological high quality of proteins, richness in vitamins and minerals and high concentration of energy (Higgs, 2000; Biesalski, 2005; Wójcik *et al.*, 2010; Pereira and Vicente, 2013; Leroy and Praet, 2015; Rohrmann and Linseisen, 2015).

Meat consumption has been accepted as a good indicator of economic and social well-being; it is a prestigious food and its consumption has increased in recent years, especially in developing countries (FAO, 2015). However, meat, like other foods, does not exclusively provide nutrients and energy. Also microorganisms, chemical residues, allergens (Taylor and Latham, 2001; OMS/FAO, 2003; WHO, 2016) and *imaginarium*, can be incorporated during a meat meal. In the present opinion article, we emphasize controversial aspects related with meat consumption: i) over consumption and chronic diseases; ii) chemical residues and cancer; iii) microorganisms and food safety. The symbolic value of meat is out of reference in this manuscript. Recently, abundant literature

data describe new chemical waste molecules in the meat subsequent to current conservation processes (drying, freezing canning smoking, salting etc.) (Baiano, 2014; Chaves-López *et al.*, 2015; Santarelli *et al.*, 2009), and cooking (grilled, baked etc.) (Seb-Choudhury *et al.*, 2014; Rohrmann and Linseisen, 2015). The consonance of this knowledge is reason enough to take into account the advice warned of Paracelsus: "*sola dosis facit venenum*". According to this ancient scholar, all things are poison and nothing is without poison, only the dosage makes the thing not poison (Paracelsus, 1965). Thus, in addition to all the controversy (to eat or not to eat meat - respectable if taken responsibly), meat consumption should comply with two fundamental pillars: the sufficient amount (following nutritional recommendations) (USDA, 2015) and hygiene enough to minimize risk of microbiological contamination (EFSA, 2015).

2. Meat consumption, a balance between good and bad.

2.1 Over consumption and chronic diseases

In this work, we assume meat definition in accordance with European Regulation (EC) N.º 853/2004, (EC, 2004). The meat consumption *per capita*, had an annual increased absolutely uncommon (Bruinsma, 2003). In undeveloped countries, according to FAO, a 2.9% meat consumption growth rate is predicted between 2005/2007 – 2030 (FAO, 2012). In developed countries, 0.6% meat consumption growth rate is expected during this interval (FAO, 2012). Despite of the recommended annual meat consumption of ≈ 33.0 kg/per capita (≈ 100g/day), in 2014 alone were consumed 76.1 kg/year/per capita in developed countries and 33.7 kg/year per capita in undeveloped countries (FAO, 2015; USDA, 2015). These data highlight the urgency of reducing meat consumption in developed countries. This average figure masks a more worrying situation affecting the undeveloped countries population presenting a large portion of their population dying of hunger or suffering from poverty with subsequent nutritional inadequacies. According to WHO (2015), metabolic disorders and cardiovascular diseases are exclusively related to the increased of the meat over consumption (OMS/FAO, 2003; Kouvari *et al.*, 2015; WHO, 2015).

2.2 Chemical residues and cancer

According to IARC, processed meat is meat that suffered alteration procedures like salting, curing, fermentation, smoking, or other processes to increase flavor or improve preservation (IARC, 2015). Recently, new molecules favoring cancer risk have been described in meat and particularly in meat products. Recent studies point out the specific role of meat components like haem iron, nitrosamines, and heterocyclic aromatic amines identified as cancer responsible (Jakszyn, 2011; IARC, 2016; Bingham, 2002; Mirvish, 1995). In addition, chemical compounds termed heterocyclic amines (HCAs) and polycyclic aromatic hidrocarbons (PAHs) were identified which are formed when muscle meat is cooked using high-temperature as takes plan during pan frying or grilling directly over an open flame (Cross and Sinha, 2004; Santarelli *et al.*, 2009; Hamidi *et al.*, 2016; Rohrmann and Linseisen, 2015; Lee *et al.*, 2016). These metabolites play an important role in carcinogenesis.

2.3. Microorganisms and food safety

According to USA official data, in the period between 1998 and 2008, meat consumption was responsible for 41.1% of bacterial, 10.8% of viral, 8.9% of chemical and 0.1% of parasitic annual domestically acquired food borne illnesses. More than 40% of bacterial food borne detected diseases were due to the ingestion of contaminated meat and poultry (Painter *et al.*, 2013). In EU, in 2013, a total of 5196 food borne outbreaks were described affecting globally 43183 human cases with 5946 hospitalizations and 11 deaths (EFSA, 2015). The main route of zoonosis transmission is diet (Harrison *et al.*, 2013). Remarkable efforts have been undertaken in European Union in order to limit the extent of foodborne infections, with the implementation of improved hygiene standards in pre and post harvest meat chain. However, the number of infections originated in ingestion of contaminated meat remains high (Rohde *et al.*, 2015; Garrido *et al.*, 2013; Dhama *et al.*, 2013).

In order to minimize the role of meat as vector of microorganisms it seems imperative: i) to implement in farms animal welfare rules with education of farmers and enforcement of existing legislation; ii) to promote a conscientious fulfilment of

the Hazard Analysis and Critical Control Point (HACCP) and Good Manufacturing Practices (GMP) and an efficient training among meat handlers (Gomes-Neves *et al.*, 2012); iii) efficient and rapid detection and tracing microorganisms in food chain by using sensitive, specific and economic tests based on DNA amplification (Fisher *et al.*, 2007). Finally, consumer education is crucial in common and practical aspects of food safety and nutritional requirements in order to ensure informed choices about food and meat choices, how to use the kitchen for cooking, the appropriate food handling and storage methods.

3. Conclusion

Eating meat may be a healthy and pleasant social and bio-cultural act. Consumer education in order to respect the recommended nutritional intake of meat in tandem with food safety practices can be expected to minimize risks related with cancer, chronic diseases and food borne illness.

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References

Baiano A (2014). Recovery of Biomolecules from Food Wastes. *Molecules,* 19: 14821-14842.

Biesalski H-K (2005). Meat as a component of a healthy diet — are there any risks or benefits ifmeat is avoided in the diet? *Meat Science,* 70: 509-524.

Bingham SA, Hughes R and Cross AJ (2002). Effect of white versus red meat on endogenous N-nitrosation in the human colon and further evidence of a dose response. *J Nutr.,* 132.

Bruinsma J (2003). Organización de las Naciones Unidas para la Agricultura y la Alimentación/londres Earthscan. An FAO perspective. *World agriculture: towards 2015/2030, Rome.*

Chaves-López C, Serio A, Mazzarrino G, Mastuscelli M, Scarpone E and Paparella A (2015). Control of household mycoflora in fermented sausages using phenolic fractions from olive mill wastewaters. *Int J Food Microbiol.,* 207: 49-56.

Cross AJ and Sinha R (2004). Mear-related mutagens/carcinogens in the etiology of colorectal cancer. *Environmental and Molecular Mutagenesis,* 44: 44-55.

Dhama K, Rajagunalan S, Chakraborty S, Verma AK, Kumar A, Tiwari R and Kapoor S (2013). Food-borne pathogens of animal origin-diagnosis, prevention, control and their zoonotic significance: a review. *Pakistan Journal of Biological Sciences,* 16: 1076-1085.

EC (2004). REG (EC) No835/2004. 2002 Regulation. EUOJ L139/55. *Journal of European Union*.

EFSA (2015a). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. European Centre for Disease Prevention and Control, European Food Safety Authority, *EFSA Journal*.

EFSA (2015b). European Food Safety Authority. http://www.efsa.europa.eu/en/press/news/151217 Accessed 01.09.2016.

FAO (2012). World agriculture towards 2030/2050 The 2012 revision. Global Perspective Studies, Agricultural Development Economics Division, *Food and Agriculture Organization of the United Nation, Rome*.

FAO (2015). FAO, Animal Production and Health. Agriculture and Consumer Protection Department. http://www.fao.org/ag/againfo/themes/es/meat/background.htm Accessed 03.09.2016.

Fisher A, Eiff C, Kuczius T, Omoe K, Peters T and Becker K (2007). A quantitative real-time immuno-PCR approach for detection of staphylococcal enterotoxins. *J Mol Med (Berl)*, 85: 461-469.

Garrido A, Chapela M, Román B, Fajardo P, Lago J and Vieites J (2013). A new multiplex real-time PCR developed method for Salmonella spp. and Listeria monocytogenes detection in food and environmental samples. *Food Control,* 30: 76-85.

Gomes-Neves E, Antunes P, Tavares A, Themudo P, Cardoso MF, Gärtner F, Costa JMC and Peixe L (2012). Salmonella cross-contamination in swine abattoirs in Portugal: Carcasses, meat and meat handlers. *International Journal of Food Microbiology,* 157: 82-87.

Hamidi EN, Hajeb P, Selamat J and Abdull Razis AF (2016). Polycyclic Aromatic Hydrocarbons (PAHs) and their Bioaccessibility in Meat: a Tool for Assessing Human Cancer Risk. *Asian Pac J Cancer Prev.,* 17: 15-23.

Harrison LM, Balan KV and Babu US (2013). Dietary Fatty Acids and Immune Response to Food-Borne Bacterial Infections. *Nutrients,* 5: 1801-1822.

Higgs JD (2000). The changing nature of red meat: 20 years of improving nutritional quality. *Trends in Food Science and Technology,* 11: 85-95.

IACR (2015). IARC Monographs evaluate consumption of red meat and processed meat. *PRESS RELEASE N° 240.* WHO, Lyon.

IARC (2016). EPIC Working Groups - Cancer working groups. http://epic.iarc.fr/research/cancerworkinggroups/bladdercancer.php Accessed 07.29.2016.

Jakszyn P, González CA, an-Barroso LL, Ros MM, Bueno-de-Mesquita HB, Roswall N, Tjønneland AM, Buchner FL, Egevad L, Overvad K, Raaschou-Nielsen O, Clavel-Chapelon F, Boutron-Ruault M, Touillaud MS, Chang-Claude J, Allen NE, Kiemeney LA, Key TJ, Kaaks R, Boeing H, Weikert S, Trichopoulou A, Oikonomou E, Zylis D, Palli D, Berrino F, Vineis P, Tumino R, Mattiello A, Peeters

PHM, Parr CL, Gram IT, Skeie G, Sánchez M-J, Larrañaga N, Ardanaz E, Navarro C, Rodríguez L, Ulmert D, Ehrnstrom R, Hallmans G, Ljungberg B, Roddam AW, Bingham SA, Khaw K, Slimani N, Boffetta PA, Jenab M, Mouw T, Michaud DS and Riboli E (2011). Red meat, dietary nitrosamines, and heme iron and risk of bladder cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC). *Epidemiol Biomarkers Prev.,* 20: 555-559.

Kouvari M, Tyrovolas S and Panagiotakos DB (2015). Red meat consumption and healthy ageing: A review. *Maturitas*, 84: 1-8. DOI: 10.1016/j.maturitas.2015.11.006.

Lee JG, Kim SY, Moon JS, Kim SH, Kang DH and Yoon HJ (2016). Effects of grilling procedures on levels of polycyclic aromatic hydrocarbons in grilled meats. *Food Chem.,* 199: 632-638. DOI: 10.1016/j.foodchem.2015.12.017.

Leroy F and Praet I (2015). Meat traditions. The co-evolution of humans and meat. *Appetite,* 90: 200-211.

Mirvish SS (1995). Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC. *Cancer Lett.,* 93: 17-48.

OMS/FAO (2003). Dieta, Nutrición y Prevención de enfermedades crónicas*.* Consulta Mixta OMS/FAO de Expertos en Régimen Alimentario, Nutrición y Prevención de Enfermedades Crónicas, *OMS/FAO, World Health Organisation*.

Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ and Griffin PM (2013). Attribution of Foodborne Illnesses, Hospitalizations, and Deaths to Food Commodities by using Outbeak Data, United States, 1998-2008. *Emerging Infectious Diseases,* 19: 407-415.

Paracelsus (1965). Die dritte Defension wegen des Chreibens der neuen Rezepte. Edited by *Werke Bd. 2. DARMSTADT: Septem Defensiones*.

Pereira PMCC and Vicente AFRB (2013). Meat nutritional composition and nutritive role in the human diet. *Meat Science,* 93: 586-592.

Rohde A, Hammerl JA, Appel B, Dieckmann R and Al Dahouk S (2015). FISHing for bacteria in food e A promising tool for the reliable detection of pathogenic bacteria? *Food Microbiology,* 46: 395-407.

Rohrmann S and Linseisen J (2015). Processed meat: the real villain? *Proc Nutr Soc.,* 1: [Epub ahead of print]. DOI: 10.1017/S0029665115004255.

Santarelli RL, Pierre F, and Corpet DE (2009). Processed meat and colorectal cancer: a review of epidemiologic and experimental evidence. *Nutr Cancer.,* 60: 131-144.

Seb-Choudhury S, Haines S, Harland D, Clerens S, van Koten C and Dyer J. Effect of Cooking on Meat Proteins: Mapping Hydrothermal Protein Modification as a Potential Indicator of Bioavailability. *J. Agric. Food Chem.,* 62: 8187-8196.

Smil V (2002). Eating meat evolution, prtterns and consequences. *Population and development review,* 28: 599-639.

Taylor LH, Latham SM and Woolhouse ME (2011). Risk factors for human disease emergence. *Philos Trans R Soc Lond B Biol Sci.,* 356: 983–989.

USDA (2015). 2015–2020 Dietary Guidelines for Americans. (8th Edn.), *USDA Publication,Washington (DC).*

WHO (2015). Q&A on the carcinogenicity of the consumption of red meat andprocessed meat. . *World Health Organization. International Agency for Research on Cancer*.

WHO (2016). Health topics, Foodborne diseases. http://www.who.int/topics/foodborne_diseases/en/ Accessed 02.25.16.

Wójcik OP, Koenig KL Zeleniuch-Jacquotte A, Costa M, and Chen Y (2010). The potential protective effects of taurine on coronary heart disease. *Atherosclerosis,* 208: 19-25.

CHAPTER II. Multiplex Real-time PCR assay to detect bacterial contamination in fresh meat.

CHAPTER II. Multiplex Real-time PCR assay to detect bacterial contamination in fresh meat.

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Abstract: Meat and meat products are important food borne source of infections. Meat consumption needs effective diagnostic tools, based on rapid, accurate and low cost technologies. In this article, we propose FiveRisks, a new diagnostic tool, based on MultiplexRT-PCRthat proved to detect a few bacteria in foodstuffs samples, like meat, cheese and fruit and vegetable juices. In just one work-day, it is possible to detect in one sample, at the same time, three food borne pathogens, *Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes*, together with*Escherichia coli* and *Staphylococcus* coagulase positive as process hygiene indicators. The specificity of the assay was proven in real samples, and is 100% and the sensitivity was, generally, in range of 10 - 10^3CFU/mL (or /g), without incubation step. With this new assay, detection of these bacteria is simply to a single PCR reaction at a very low cost.

Multiplex Real Time PCR Assay to Detect Bacterial Contamination in fresh meat.

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Abstract

Bacterial contamination among foodstuffs remains at high levels, particularly in Europe, despite regulatory efforts to address the situation. The need of a new hygiene practices and diagnostic tools are crucial. Ideally, such tests would be facile, accurate, and inexpensive to be used as a first line detection of bacterial contamination at meat plants. In this sense, a multiplex real-time PCR has been developed for identification of bacterial pathogens and bacterial hygiene indicators at meat plant level. Were present and discuss data obtained in order to its validation. The limit of detection was determined as 10 CFU/g of meat for *Salmonella* spp., and *Escherichia coli*, 10^3 CFU/g for *Campylobacter* spp., 10^6CFU/g for *Listeria monocytogenes* and 10^4CFU/g meat for *Staphylococcus* coagulase positive, without an enrichment step. Our proposal simplifies bacterial detection and reduces costs per test.

Keywords: Multiplex Real-Time PCR; Fresh meat; Bacterial contamination**.**

1. Introduction

Compelling evidence of cross contamination between carcasses and meat entering the food has been provided, supporting the hypothesis that contamination is transferred between successive phases of, at least, pig abattoir and processing (Swanenburg et al., 2001, Lo Fo Wong et al., 2002; Botteldoorn et al., 2004, Vieira-Pinto et al., 2005, Vieira-Pinto et al., 2006 and De Busser et al., 2011). Unequivocally, Gomes–Neves et al., (2012), indicate that pork is an important source of *Salmonella* and those procedures generally in place at present abattoir procedure could promote its contamination. Although swine can harbor *Salmonella* before slaughter, the abattoir environment can contribute to further cross-contamination along the processing line, including contact with meat handlers. Intriguingly, other investigators have pointed out that primary production phase and the slaughterhouse environment are the main sources of the contamination of carcasses and meat (Botteldoorn et al., 2004, Vieira-Pinto et al., 2005, Vieira-Pinto et al., 2006, Delhalle et al., 2009 and De Busser et al., 2011). Of more concern, the abattoir environment and the slaughter operations seem not only to harbor multiple drug resistance *Salmonella* serotypes originated in the pig reservoir, but

also propagate them through cross-contamination processes, involving meat handlers (Gomes-Neves et al., 2014). Measures are needed to improve standards in the post-harvest pork meat chain, in order to moderate the prevalence of *Salmonella* positive pigs at the primary production phase and to decrease sources of contamination at the abattoir (De Busser et al., 2011). The need to invest in general hygiene improvement, training for meat handlers, good manufacturing practice and HACCP implementation (Lo Fo Wong et al., 2002, Delhalle et al., 2009, and Gomes-Neves et al., 2011), all remain crucial to reduce cross-contamination and to minimize levels of contamination to as low as possible. Meanwhile, new practices are required at meat plants and meat industry. *Salmonella* spp. and *Campylobacter* spp. and the *Listeria monocytogenes* dominate as the major food-borne bacterial pathogens reported by EFSA. Cultural methods remain as golden standard tests for bacterial detection. However, these practices are time consuming and require high-qualified professionals. According to Fisher, 2007, the emergence of a new generation of methods based on DNA amplification need to be developed rapid tests for bacterial identification in foodstuffs. In this sense, a new multiplex real-time PCR has been developed as a qualitative method for identification of bacteria: i) pathogens (*Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes*) and ii) hygiene indicators (*Escherichia coli* and *Staphylococcus* coagulase positive). Here, we present and discuss data obtained in order to validate the 'FiveRisks' test, which we propose as an accurate and useful assay for bacterial control in the fresh meat food chain.

2. Material and Methods

2.1 Bacterial Reference material

The reference material was obtained from Spanish Type Culture Collection (CECT), private collections (PC) and Ielab commercial collection(Table 1).Preparation of reference stock and working culture was performed according to ISO 11333: 2014. Reference strains stock was freezing at a temperature below −70°C for *Campylobacter*spp. and below−20 °C for the other microorganism (Table 1).

2.2 Preparation of test inoculum

Using a sterile loop, *Campylobacter*spp. was transferred to Brucella Broth and incubated in micro-aerobic atmosphere, during 48h at 37ºC and then plated on Columbia blood agar base and incubate a 37°C, 48h in micro-aerobic atmosphere.Other bacteria were plated onTryptone soy agar (TSA) and incubated at 37°C,24h in aerobic conditions. These fresh cultures were then transferred to Brain-Heart Infusion Broth (BHI) and incubated during 24h at 37ºC in aerobic conditions, except for *Campylobacter* spp., this one on Brucella broth and incubated in micro-aerobic atmosphere. To estimate the number of bacteria, decimal dilutions of each strain were performed, followed byplating on TSA and incubated during 24h at 37ºC on blood Colombia agar base in micro-aerobic atmosphere during 48h at 37ºC. The concentration of the working culture was for: $1.5x10^9$colony-forming unit per mL(CFU/mL) for *Salmonella* spp., $2.1x10^9$CFU/mL for *L. monocytogenes*, from 10^5 CFU/mL for *Campylobacter* spp., $1.7x10^9$CFU/mL for *Staphylococcus* coagulase positive and $2.8x10^9$CFU/mLfor *Escherichia coli*.

2.3. Primers and probes design

Five discrete sets of primers were designed based on sequences downloaded from the NCBI GeneBank ® (NCBI, 2013). Primers were designed using the Primer Express 2.0.0 Software program and were synthesized by NZYTech® Company (Portugal). We evaluated the use of *gapA* gene, *hylA* gene, *cdtB* gene, 16S RNA and *uspA* gene to detect *Salmonella* spp., *L. monocytogenes*, *Campylobacter* spp., *S.* coagulase positive and *E. coli*, respectively. We choose the *gapA* gene to detect *Salmonella* spp., because this is a housekeeping gene, conserved across the salmonellae (McQuiston et al., 2008). To detect *L. monocytogenes,* was tested *hlyA* gene; this gene codify listeriolysin O (LLO), present only in virulent strains of the species and is required for virulence (Churchill et al., 2006; Blais et al., 1997, Hough et al., 2002, Hudson et al., 2001, Lehner et al., 1999, Lunge et al., 2002 and Rodríguez-Lázaro et al., 2004). The *Campylobacter* spp. detection has been done targeting the *cdtB* gene, which is co-responsible for the production of a virulence factor, a cytolethal distending toxin subunit (CDT) (Plckett et al., 1996 and Asakura et al., 2008). For *S.* coagulase positive detection, it was targeted a conserved region of its 16S rRNA gene. To

detect *E. coli* strains, *uspA* gene was chosen because it encodes the universal stress protein and was described to be highly specific for pathogenic and non-pathogenic *E. coli* stains (Chen & Griffiths, 1998). Primers were designed using the Primer Express 2.0.0 Software program and were synthesized by NZYTech® Company (Portugal).

Probe sequences were designed using the Software Fast PCR 6.5 and were confirmed by using the Beacon Design™ Software 8.13 and synthesized by Eurofins Genomics (Germany) (to safeguard the intellectual property, the primers and probes sequences of FiveRisk will not be disclosed).

The specificity of primers was evaluated by using Blast® NCBI Software on-line program and it was confirmed by real-time PCR with CECT reference bacteria strains; briefly, each set of primers and probes of microorganisms was subjected to amplification over the DNA; 10x dilutions of genomic DNA extracted from a bacterial culture, in limit dilution approach for all monoplex reactions. Plasmids containing the synthetic target gene of each strain were cloned in the plasmid vector *pUC57* (genes and clones were synthesized commercially by NZYTech® Company (Portugal)). These plasmids and respective dilutions were subject to real time-PCR until no signal was detected, at a limit dilution approach. Subsequently, three plasmid concentrations were tested in the next steps: the plasmid concentration of the limit dilution achieved, at 10x concentrated plasmid solution, and at 10x diluted plasmid solution. Finally, the accuracy of this assay was evaluated with spiked samples.

2.4. Multiplex real-time PCR conditions

The monoplex and multiplex real-time PCR assay were delineated using synthetic plasmids as positive control and DNase free distilled water as no-template control (NTC). Amplification was performed by using 21µL iQ™Multiplex Powermix (2X) (Bio rad), 300µM of primer mix, 300 µM of probes, 1µL of DNA template and 1µM of iTaq™ DNA Polymerase (5 U/µL), in a 41µL reaction. The real-time PCR was performed under the following conditions: 5 min at 95ºC; 30 cycles of 20 sec at 95ºC, 20 sec at 56ºC, 20 sec at 72ºC; and a final extension step of 5 min at 72ºC.

2.5. Spiked samples

For spiked samples preparation, we used pork meat (P-1), fresh pasteurized cheese (PC-1), and green juice conserved by High Pressure Processing Technology (HPP) (GJ-1). Ten grams of each sample was homogenized in 85 mL of Peptone Water (BPW-F 225mL bioMérieux), added to a stomacher bag (Tempo®SACS bioMérieux) and homogenized during 1 minute at medium pressure in a Stomacher® 400 (Seward). For control samples the procedure was similar added with 90 mL of Peptone Water (BPW-F 225 mL bioMérieux). The foodstuffs were spiked with 1 mL of each of five cultures previously prepared, four of them in BHI (*Salmonella* spp., E. *coli*, *S.* coagulase positive and *L. monocytogenes*) and one in Brucella Broth (*Campylobacter* spp.) with microbial load referred above. The mix was homogenized for 1 min. Aftertwo samples of 1 mL were collected of each sample, P-1, PC-1 and GJ-1 with, one was submitted to DNA extraction and another one was used for ten serial dilutions (10X). For DNA extraction a QIAamp® DNA Mini and Blood Mini Handbook kit (QIAGEN) was used, according to the protocol for isolation of genomic DNA from Gram-positive bacteria. For PCR reactions, we use 1µL of DNA template, 1µL of plasmids as positive control, and DNase free distilled water as NTC.

3. Results

3.1. Sensitivity and specificity of Multiplex real-time PCR

The specificity of primers and probes by using the BLAST algorithm were: to all pair of primers and probes, the maximum identity obtained was 100%. However, the query coverage was 100% for *Salmonella* spp. and *Campylobacter* spp., and 65% for *L. monocytogenes*. All pairs of primers presented an E-value greater than 0.01 (Altschul et al., 1990).

Table 1 provides the findings for sensitivity of the Multiplexreal-time PCRassay. According to Figure 1 a) to e), five calibration curves have been defined with a mix of all pairs of primers and probes, starting with a concentration of 2µg in 20µL. Once the limit dilution for each bacterium was achieved, according

to Table 1 and Figure 1 f) to h) it has been made three mix to define the limit dilution assay.

Table 1. Test panel for specific detection of *Salmonella* spp., *E. coli*, *L. monocytogenes*, *Campylobacter* spp. and *S.* coagulase positive; Limit detection of all bacteria in a singleplex reaction and in a multiplex reaction with synthetic plasmids (ug/uL) and limit detection of all bacteria (cfu/g) in a multiplex reaction with spiked samples.

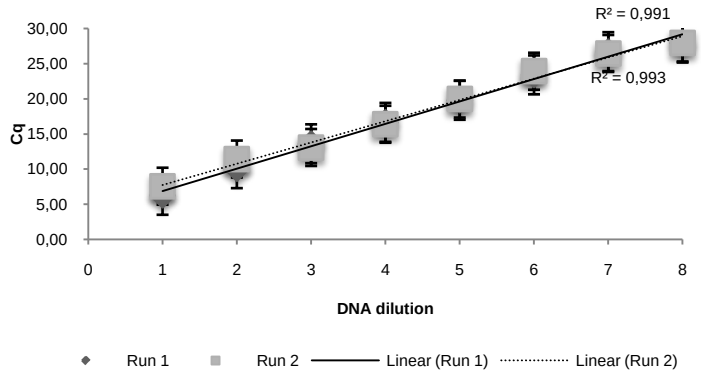
Bacteria	**CECT and PC Reference DNA**	**Ielab commercial collection**	**LIMIT OF DETECTION**				
			Plasmid concentration in a singleplex reaction (ug/uL)	**Plasmid concentration in a multiplex reaction (ug/uL)**	**Spiked sample Meat P-1 (UFC/g)**	**Spiked sample Cheese PC-1 (UFC/g)**	**Spiked sample Juice GJ-1 (UFC/mL)**
Salmonella spp.	*S.enterica* serotip Budapest (PC)	NCTC 6017 / NTCC 7832	10^{-8}	10^{-7}	10	10	10^{2}
Escherichia coli	ATCC 23231	ATCC 25922	10^{-8}	10^{-7}	10	10	10
Listeria monocytogenes	ATCC 19114	ATCC 13932	10^{-8}	10^{-7}	10^{6}	10^{6}	10^{6}
Campylobacter spp.	ATCC 33291	ATCC 33291	10^{-8}	10^{-7}	10^{3}	10^{3}	10^{4}
Staphylococcus coagulase positive	ATCC 6538	ATCC 25923	10^{-6}	10^{-5}	10^{4}	10^{4}	10^{5}

3.2. Comparison of a multiplex assays efficiency results when combining all DNA's versus the use of DNA from a single strain

The performance of multiplex assay was evaluated according to: i) a single plasmid in reaction with all primers and probes (Figure 1 a) to e)), and ii) all plasmids in reaction with all primers and probes (Figure 1 f) to h)). As we can observe, both reactions present similar performances. However, *S.* coagulase positive and *L. monocytogenes*, amplification was slightly reduced. Plasmids and spiked samples analysis were executed in replicates in order to minimize intra-assay variability. Figure 1 presents evidences of universal fluctuations that do not produce obvious distortions in the reported signal, but they do affect the precision of replicates. To ensure the accuracy of these replicates, we calculated the correlation coefficient (R^2). This value reflects the linearity of the standard curve and, according to scientific literature, ideally, $R^2 = 1$, although 0,999 is generally the maximum value accepted. As shown in Figure 1 a) to e), the R^2 minimum is 0.983 and the R^2 maximum is 0.999. These results suggest robustness and accuracy of our proposal.

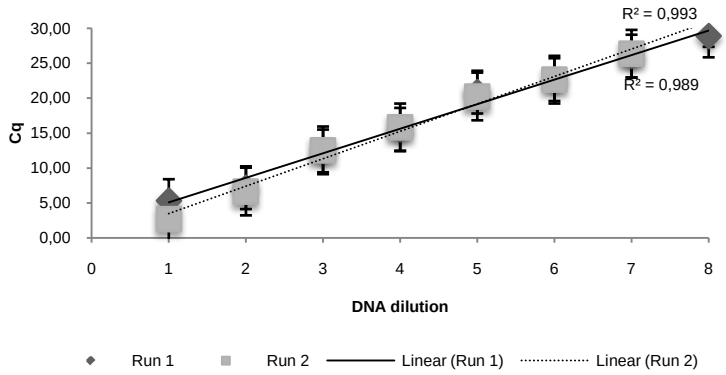
a1) *Salmonella* spp. mRT-PCR results

a2) *Salmonella* spp. standard curve



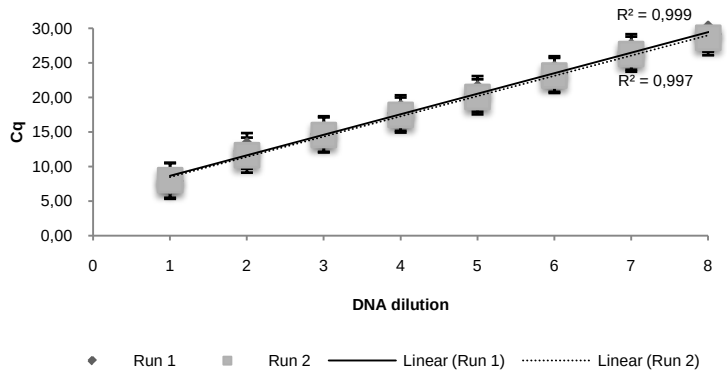
b1) *Listeria monocytogenes* mRT-PCR results

b2) *Listeria monocytogenes* standard curve



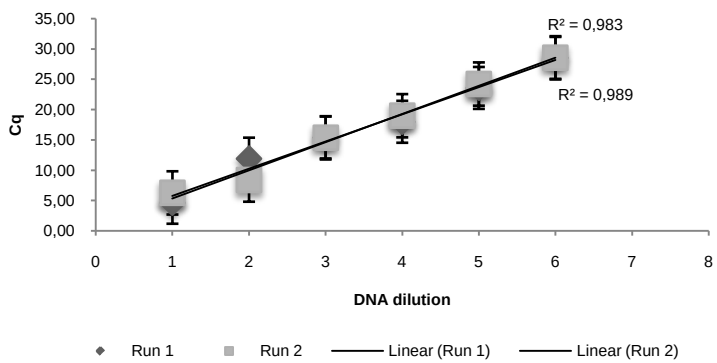
c1) *Campylobacter* spp. mRT-PCR results

c2) *Campylobacter* spp. standard curve



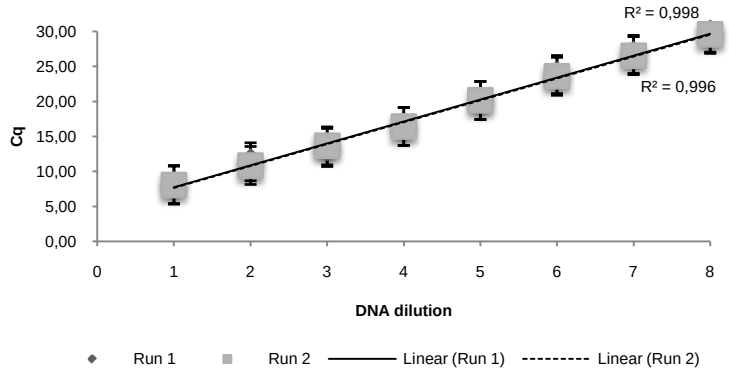
d1) *Staphylococcus* coagulase positive mRT-PCR results

d2) *Staphylococcus* coagulase positive standard curve



e1) *Escherichia coli* mRT-PCR results

e2) *Escherichia coli* standard curve



f) Plasmid concentration of the limit dilution achieved

g) 10x concentrated plasmid solution

h) 10x diluted plasmid solution

1

Figure 1.DNA provided by reference strains used in spiked samples a1) *Salmonella* spp., mRT-PCR results; a2) *Salmonella* spp. standard blue curve b1) *L. monocytogenes* mRT-PCR results; b2) *L. monocytogenes* standard purple curve c1) *Campylobacter* spp., mRT-PCR results; c2) *Campylobacter* spp. standard brown curve d1) *S.* coagulase positive, mRT-PCR results; d2) *S.* coagulase positive standard green curve e1) *E. coli* mRT-PCR results; e2) *E. coli* standard red curve. Multiplex Real-time PCR profiles obtained for all 5 synthetic plasmids tested with the mix of all pairs of primers and probes: f) the plasmid concentration of the limit dilution achieved g) 10x concentrated plasmid solution h) 10x diluted plasmid solution. Blue curves – *Salmonella* spp., Red curves – *E. coli*, Green curves – *S.* coagulase positive, Purple curve – *L. monocytogenes*, Brown curve – *Campylobacter* spp.

3.3. Spiked samples study by Multiplex real-time PCR

We determined the limit of detection of the assay, for all bacteria. As expected, we observed that this limit varied according to the food matrix and the bacteria (Table 1). The sensitivity (limit of DNA detection) of the assay is in the range of 10– 10^3 CFU/g). For *S.* coagulase positive, the limit of detection is ~10^4 CFU/g of sample in the vegetable matrix. DNA extraction method was the same for every sample; however, the amplification process presents dissimilarities, probably related with the DNA extraction accuracy or known protein/proteinase inhibitors in foodstuffs, enzymes with heme groups in meat samples, lactose and sugars in dairy products or polyphenols in vegetables, and so forth all of which can inhibit PCR (Schrader et al., 2012). Nonetheless, Multiplexreal-time PCRwas definitely sufficiently accurate for detection of low levels of bacterial contamination: 10CFU/g of meat for *Salmonella* spp., and *E. coli*, 10^3CFU/g meat for *Campylobacter* spp., 10^4CFU/g meat for *S.* coagulase positive and 10^6CFU/g meat for *L. monocytogenes*.

4. Discussion

Microbes, their toxins, and allergens in food account for about 23 million episodes of illness and 5,000 deaths in Europe every year (EFSA, 2015). Consensually, the requirements need to attain higher standard of hygiene are complex; they includes measures at the primary production phase, at the abattoir, and in the post-harvest phase in order to reduce the prevalence of pathogens in animal production and to decrease sources of contamination at the abattoir (De Busser et al., 2011). In addition, there is a need to invest in improvement of general hygiene, to upgrade training for meat handlers, for good manufacturing practice and for the urgent implementation of HACCP (Lo Fo Wong et al., 2002, Delhalle et al., 2009 and Gomes-Neves et al., 2011). The present work describes aMultiplex real-time PCRspecifically oriented to study bacterial contamination on foodstuffs, including fresh meat samples. Our proposal – FiveRisks - is a first line test for bacterial contamination at large for use at meat plants, as: i) is facile, accurate and inexpensive, and, ii) is not oriented to only one specific pathogen.

According to our results no conflict or inhibitory action was verified on specific DNA amplification, even at low level of DNA concentration. In limit, this evidence was considered our internal and unequivocal control for validation of specific strain DNA amplification. Also, the procedure is able to detect, without enrichment, bacteria at 10 -10^3 CFU/g foodstuff samples, major indicator of excellent performance. So, the inclusion of an enrichment step prior to DNA extractions and amplification in order to ameliorate the performance of FiveRisks, the ability to detect lower burden of bacteria in foodstuffs, is under evaluation.

Finally, FiveRisks is a methods based on DNA amplification. We recommend that positive cases must be oriented for confirmation of the presence de viable pathogens, at the national reference laboratory.

Conflict of interests

We declare no competing interests.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990). Basic local alignment search tool. *J Mol Biol*, *215*, 403-410.

Asakura M., Samosornsuk W., Hinenoya A., Misawa N., Nishimura K., Matsuhisa A. & Yamasaki S. (2008). Development of a cytolethal distending toxin (cdt) gene-based species-specific multiplex PCR assay for the detection and identification of Campylobacter jejuni, Campylobacter coli and Campylobacter fetus. *FEMS Immunol. Me. Microbiol,52*, 260-266.

Blais, B.W., Turner, G., Sooknanan, R. & Malek, L.T. (1997). A nucelic acid sequence-based amplification system for detection of Listeria monocytogenes hlyA sequences. *Applied environmental microbiology,63*, 310-313.

Botteldoorn, N., Herman, L., Rijpens, N. & Heyndrickx, M. (2004). Phenotypic and molecular typing of Salmonella strains reveals different contamination sources in two commercial pig slaughterhouses. *Applied and Environmental Microbiology, 70,* 5305-5314.

Chen, J. & Griffiths, M.W. (1998). PCR differentiation of Escherichia coli from other Gramnegative bacteria using primers derived from the nucleotide sequences flanking the gene encoding the universal stress protein. *Letters in Applied Microbiology,27*, 369-371.

Churchill, R.L.T., Lee, H. & Hall, J.C., (2006). Detection of Listeria monocytogenes and the toxin listeriolysin O in food. *Journal of Microbiological Methods, 64,* 141-170.

De Busser, E.V., Maes,D., Houf, K., Dewulf, J., Imberechts,H., Bertrand, S. & De Zutter, L. (2011). Detection and characterization of Salmonella in lairage, on pig carcasses and intestines in five slaughterhouses. *International Journal of Food Microbiology, 145,* 279-286.

Delhalle, L., Saegerman, C., Farnir, F., Korsak, N., Maes, D., Messens, W., De Sadeleer, L., De Zutter, L. & Daube, G. (2009). Salmonella surveillance and control at post-harvest in the Belgian pork meat chain. *Food Microbiology, 26,* 265-271.

European Food Safety Authority. (2015). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. European Centre for Disease Prevention and Control, *EFSA Journal*.

Gomes-Neves E., Cardoso, C.S., Araújo, A.C. & Costa, J.M.C (2011). Meat handlers training in Portugal: A survey on lnowledge and practice. *Food Control, 22,* 501-507.

Gomes-Neves, E., Antunes, P., Manageiro, V., Gärtner, F., Caniça, M., Costa, J.M.C. & Peixe, L. (2014). Clinically relevant multidrug resistant Salmonella enterica in swine and meat handlers at the abattoir. *Veterinary microbiology, 168,* 229-233.

Gomes-Neves, E., Antunes, P., Tavares, A., Themudo, P., Cardoso, M.F., Gärtner, F., Costa, J.M.C. & Peixe, L. (2012). Salmonella cross-contamination in swine abattoirs in Portugal: Carcasses, meat and meat handlers. *International Journal of Food Microbiology, 157*, 82-87.

Hough, A.J., Harbison, S.-A., Savill, M.G., Melton, L.D. & Fletcher, G. (2002). Rapid enumeration of Listeria monocytogenes in artificially contaminated cabbage using real time polymerase chain reaction. *Journal of food protection, 65,* 1329-1332.

Hudson, J.A., Lake, R.J., Savill, M.G., Scholes, P. & McCormick, R.E. (2001). Rapid detection of Listeria monocytogenes in ham samples using immunomagnetic separation followed by polymerase chain reaction. *Journal of applied microbiology, 90,* 614-621.

Lehner, A., Loncarevic, S., Wagner, M., Kreike, J. & Brandl, E. (1999). A rapid differentiation of Listeria monocytogenes by use of PCR-SSCP in the listeriolysin O (hlyA) locus. *Journal of microbiological methods, 34,* 165-171.

Lo Fo Wong, D.M., Hald, T., van Der Wolf, P.J. & Swanenburg, M. (2002). Epidemiology and control measures for Salmonella in pigs and pork. *Livestock Production Science, 76,* 215-222.

Lunge, V.R., Miller, B.J., Livak, K.J. & Batt, C.A. (2002). Factors affecting the performance of 5' nuclease PCR assays for Listeria monocytogenes detection. *Journal of microbiological methods, 51,* 361-368.

McQuiston, J.R., Herrera-Leon, S., Wertheim, B.C., Doyle, J., Fields, P.I., Tauxe, R.V. & Logsdon Jr, J.R. (2008). Molecular Phylogeny of the Salmonellae: Relationships among Salmonella Species and Subspecies Determined from Four Housekeeping Genes and Evidence of Lateral Gene Transfer Events. *American Society for Microbiology, 190,* 7060-7067.

NCBI - National Center for Biotechnology Information. Genetic Sequence Data Bank. (2013) http://www.ncbi.nlm.nih.gov/genebank/ (accessed 07.03. 13).

Plckett, C.L., Pescl, E.C., Cottle, D.L., Russell, G., Erdem, A.N. & Zeytln, H. (1996). Prevalence of cytolethal distending toxin production in Campylobacter jejuni and relatedness of Campylobacter sp. cdtB gene. *Infection and Immunity, 64,* 2070-2078.

Rodríguez-Lázaro, D., Hernández, M., Scortti, M., Esteve, T., Vazquez-Boland, J.-A. & Pla, M. (2004). Quantitative detection of Listeria monocytogenes and Listeria innocua by real-time PCR: assessment of hly, iap, and lin02483 targets and AmpliFluor technology. *Applied environmental microbiology, 70,* 1366-1373.

Schrader, C., Schielke, A., Ellerbroek, L. & Johne, R. (2012). PCR inhibitors - occurrence, properties and removal. *Journal of applied microbiology, 113,* 1014-26.

Swanenburg, M., Urlings, H.A.P., Snijders, J.M.A., Keuzenkamp, D.A. & Van Knapen, F. (2001). Salmonella in slaughter pigs: prevalence, serotypes and critical control points during slaughter in two slaughterhouses. *International Journal of Food Microbiology, 70,* 245-256.

Vieira-Pinto, M., Tenreiro, R. & Martins, C. (2006). Unveiling contamination sources and dissemination routes of Salmonella sp. in pigs at a Portuguese slaughterhouse through macrorestriction profiling by pulsed-field gel electrophoresis. *International Journal of Food Microbiology, 110,* 77-84.

Vieira-Pinto, M., Themudo, P. & Martins, C. (2005). Occurrence of Salmonella in the ileum, ileocolic lymph nodes, tonsils, mandibular lymph nodes and carcasses of pigs slaughtered for consumption. *J Vet Med B Infect Dis Vet Public Health, 52,* 476-481.

World Health Organization. (2015). WHO estimates of the global burden of foodborne diseases: foodborne disease burden epidemiology reference group 2007-2015. Geneva: WHO Press.

CHAPTER III. Food safety: an evaluation of Portuguese butcher shops.

CHAPTER III. Food safety: an evaluation of Portuguese butcher shops.

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Abstract: The main objective of this paper was to get some insight into the target markets for FiveRisks and to fill the knowledge gap in central areas of the meat chain. We did a questionnaire to evaluate 73 butcher shops in Center of Portugal, to determine some relations between training and compliance with the food safety European and Portuguese law. Firstly we did a brief demographic description, an evaluation of the type of training and the impact of that training in the good handling practices. Then, on finally, we did a visual evaluation of the maintenance of thefacilities, to understand if it accomplishes the European law. The results have shownthat it is important to improve the level of the training and increasing the requirement and knowledge levels related to the training imposed by the European law. It is also essential that the competent authorities have an accurate assessment of the knowledge acquired in the context of professional training. This check is a critical point to control cross-contamination risks in meat chain.

Meat safety: an evaluation of Portuguese butcher shops.

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Abstract

Butcher shops are end points in the meat chain and they can have a determinant role in cross-contamination control. This study aims to determine whether Portuguese butcher shops comply with European and Portuguese law regarding the sale of fresh meat and meat products. An assessment of the hygiene of the meat handlers and the facilities and maintenance of premises was carried out (n=73). Handlers (n = 88) were presented with a questionnaire composed of “Knowledge” and “Practice” questions about Hazard Analysis and Critical Control Point (HACCP) and Good Practice in Food Industry (GPFI), to assess knowledge and compliance of food safety practices. A checklist composed of 27 items was used to evaluate the hygiene of facilities and meat handlers and the maintenance of the butcher shops. Our results revealed some lack of compliance in all the topics evaluated .The mean “Knowledge” and “Practice” score among the operators was 68.0% and the mean “visual inspection” score for the butcher shops was 64.0%. Severe deficiencies were observed regarding the mandatory implementation of HACCP principles in this type of small food businesses. The findings indicate a need to modify training to enhance compliance of European food safety regulations at this step of the meat chain.

Keywords: food safety; butcher shops; meat handlers; hygienic conditions.

Introduction

The European Union policy has clearly demonstrated a particular concern for food safety and the supply chain quality*(44)*, which is directly related to the Common Agricultural Policy, sustainable development and Public Health *(26)*. In addition, and whereas safe food is a basic human right, thousands of Europeans are affected annually by episodes of foodborne disease*(26, 15)*. Foodborne infections due to the consumption of contaminated food, particularly meat and meat products, are considered a public health problem. Moreover, it is increasingly important to identify at what stage of the food chain the contamination occurs and how cross-contamination can be prevented or eliminated in the subsequent stages*(20, 21)*.

In the meat sector, the European Commission (EC) has developed an approach to minimize this problem in production, distribution and at the point of sale. Different regulations were approved regarding specific hygiene instructions for food hygiene rules for food of animal origin, microbiological criteria for foodstuffs and regulations for animal by-products*(14, 13, 12, 11)*. The main objective of this set of regulations was to increase the level of operator education as a mean to prevent foodborne illnesses *(35, 25, 4, 44)*. In Portugal, beyond the European legislation currently in place, the hygienic conditions and the techniques to monitor the distribution and sale of meat and meat productshave been defined*(9)*. In parallel, the Facilities Monitoring and Control Plan (PACE) was created by the Portuguese veterinary authority. The main objective of this plan was to establish criteria necessary for official inspections and audits supported by different checklists: one of these isspecifically for butcher shops. This plan intends to maintain food safety and public health, and promote adherence to the Portuguese, European and international hygiene regulations*(8)*. As training for meat handlers is considered essential, a specific Portuguese law was enacted to regulate a “handler card”*(10)*. This is mandatory for personnelworking in the meat retail businesses. To obtain this card, the professional must attend 15 hours of mandatory training in diverse subjects. The topic of this training cover meat hygiene, food microbiology, personal hygiene for meat handlers, and hygiene in working spaces and for equipment, packaging of meat and meat products, hygiene at the point of sale of the meat selling and delivery, food safety and Hazard Analysis and Critical Control Point (HACCP) and work safety and hygiene. (WSH)*(27, 10)*. In Portugal, despite the implementation of legal measures which theoretically should lead to an improvement in the safety of food products available to the consumer, there are no unequivocal data on the implementation of good practices in the meat processing and retail sale sectors. To our knowledge, only a few studies have focused on meat chain steps, namely in abattoirs*(27)*, and butcher shops *(42, 28)*.

The aim of this study was to assess knowledge levels of meat handlers in butcher shops concerning food safety knowledge practices and to determine the level of compliance with EU food law compliance in the maintenance and the hygiene of the facilities and the personal hygiene of the meat handlers.

Material and methods

Questionnaire design and delivery

The questionnaire was based on a Portuguese meat handlers' study developed by Gomes-Neves and colleagues (2011) *(27)*. The survey included 23 multiple-choice questions, divided into three sets, with two to five possible answers depending on the topic. The first set of questions dealt with "general evaluation" questions and included: age, gender, marital status, level of education, years of relevant experience, training in Good Practice in Food Industry (GPFI) and training in WSH. The second set included three "knowledge" questions; the goal of this set was to ascertain levels of general knowledge of professionals in food safety, definition of HACCP, symptoms associated with food poisoning and risk groups for foodborne diseases. Within this set of questions, two had the option of an answer of "do not know", with the purpose of minimizing the social desirability *(40)*. The third section, which consisted of 13 questions denominated "Practice" analyzed the GPFI in butcher shops.

The questionnaire was conducted in person, in 73 local independent and city market butcher shops in Central Portugal over eight months from September 2015 to April 2016. Questionnaires were randomly assigned and were distributed as described below to reduce negative impact of conducting the study on the work of meat handlers'.In shops with one or two meat handlers, one questionnaire was conducted, whereas in shops with three or more meat handlers, more than one questionnaire was carried out. For each participant the proportion of correct answers was calculated, and a score was considered that could range from 0.0%, when there were no correct answers, to 100.0% when all answers were correct. The participants who answered the questionnaire have remained anonymous: each participant was informed of the survey's purpose and that confidentiality would be assured.

Checklist design and assessment

A checklist with 27 items was used to evaluate the hygiene and the maintenance of facilities (pest control systems, meat exposition, cleaning cloths or

detergents, presence of cleaning materials, HACCP prerequisites) and operators(personal hygiene and use of personal protective equipment). For each butcher shop the proportion of positively checked items was calculated. This proportion was considered a score that could range from 0.0% when there was no positively checked item to 100.0% when all items were considered correct according to the law.

Statistical analysis

Categorical variables were described as percentages. Score results were given as minimum, maximum and mean. Microsoft® Excel® for Mac™© 2011. Version 14.1.0 was used to analyze the information obtained with the questionnaires.

Results

Demographic profile of the meat handlers and analysis of training areas

Answers were obtained from all the butcher shops contacted. There were 88 participants, 26 females and 62 males. The general characteristics of the participants are provided Table 1. Butchery is a job usually performed by men (70.5%), with limited years of education. More than half of those interviewed (56.8%) were 45 years of age or older and most participants had more than five years of relevant experience (93.2%). Two different training areas were evaluated, GPFI and WSH and almost all of those interviewed had professional training in these areas (97.7%), and in the majority during the previous year (62.5%).

Table 1 Socio-demographic characteristics and job information of the participants (N=88).

Variable	Answers	%	n
Age	18 - 23 years	1.1	1
	24 - 30 years	4.5	4
	31 - 45 years	37.5	33
	> 45 years	56.8	50

Gender	Male	70.5	62
	Female	29.5	26
Marital Status	Single	10.2	9
	Married/Consensual union	83.0	73
	Widower	2.3	2
	Divorced	4.5	4
Years of formal education	Primary education	26.1	23
	Basic and undergraduate studies	69.3	61
	Higher education	4.5	4
Years of relevant experience	< 1 year	2.3	2
	1-3 years	2.3	2
	3-5 years	2.3	2
	> 5 years	93.2	82
Training in good practice on food industry	Yes	97.7	86
	No	2.3	2
Training in work safety and work hygiene	Yes	97.7	86
	No	2.3	2
Last training	Last year	62.5	55
	Two years ago	28.4	25
	More than three years ago	9.1	8
	Not have training	0.0	0

Questionnaire analysis

The mean “Knowledge” and “Practice” score among operators was 68.0%, with 43.0% as minimum and 93.0% as maximum.

“Knowledge” and “Practice” questions

Despite implementation of HACCP by all the butcher shops, less than half of the meat handlers (46.7%) identified correctly the definition. Only about one quarter of the respondents (23.9%) was able identify correctly the risk group for foodborne diseases. On the other hand, most participants (94.3%) recognized the principal symptoms of foodborne illnesses (Table 2).

Table 2 Frequencies of answers to “knowledge” questions (N=88).

Question	Answers	%	n
Identification of risk groups for foodborne diseases	No one	3.4	3
	Old people, children and patients with immunodeficiency	23.9	21
	General public	60.2	53
	I don't know	12.5	11
Identification of common symptoms of foodborne disease caused by contaminated beef	Bleeding	0.0	0
	Body pain	0.0	0
	Abdominal pain, fever, vomit and diarrhea	94.3	83
	I don't know	5.7	5
HACCP definition	Is a new community legislation	30.7	27
	Is a control of hazards in food production	46.6	41
	Is a certification method	20.5	18
	I do not know what is HACCP, but I have heard.	2.27	2
	Never encountered	0.00	0

Concerning the questions dealing with “Practice”, all operators reported that the temperature at which meat was stored was checked daily. When asked if they use different knives for preparing meat of poultry, pork and beef as the law requires, half of the respondents (48.9%) answered positively but thirty five point two percent (35.2%) reported the use of the same knife. About one third (30.7%) of the respondents claimed that they used different cutting surfaces depending on the meat species. As the questionnaire was self-reported these results can be higher because meat handlers might have omitted this information. When questioned if minced meat was all prepared in the same mincer, irrespective of whether it was beef, pork or poultry, sixty percent (60.2%) of the participants responded positively, implying the absence of separation of poultry meat from beef/pork, as required by Portuguese law. In addition, based on the answers from

participants, about three quarters of the meat handlers (72.7%) fail to separate different types of fresh meat and/or meat products (e.g. smoked sausages) into different bags in the moment of the sale. In regard to visible signs of damage in meat, more than eighty percent (84.1%) of the participants noted that they the affected portion, according to Commission Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21 October 2009 (11) (Table 3).

Questionnaire results, analysis – "Practice" questions about butcher shops characteristics

All butcher shops visited in the survey used the public water supply (100.0%). Despite the fact that seventy three point nine percent (73.9%) had the support of an external company in order to implement and verify HACCP, only around half (48.0%) had an animal by-products external operator responsible for the removal and transport which is a requirement of the EU law. A small minority of the participants (5.7%) did not have an animal by-products container. In spite of these irregularities, seventy two point seven percent (72.7%) of the butcher shops included here produced meat products (Table 3).

Table 3 Frequencies of answers to "Practice" questions (N=88).

Question	Answers	%	n
Use of different knives for handling meat from different animal species	Yes	48.9	43
	No	35.2	31
	Whenever possible	15.9	14
Meat preparation in the same workbench	Yes	52.3	46
	Species are prepared separately	30.7	27
	No, but is random	17.1	15
Meat minced in the same mincer	Yes	60.2	53
	Mince poultry meat on another mincer	34.1	30
	Although they had more than one mincer, use these randomly	5.7	5

Type of water	Public water supply	100.0	88
	Another	0.0	0
Storing temperaturedaily checked	Yes	100.0	88
	No	0.0	0
Different types of fresh meat and/or meat products placed on the same bag	Yes	23.9	21
	No	72.7	64
	Whenever possible	3.4	3
Procedure to remove meat portions with clear signs of damage:	Remove the meat portion	84.1	74
	Remove the meat portion for the general waste	11.4	10
	I don't know how to proceed	3.4	3
	Wait indications provided by the responsible person	1.1	1
Hygiene plan	Yes	100.0	88
	No	0.0	0
External HACCP support company	Yes	73.9	65
	No	26.1	23
Animal by-products container	Yes	94.3	83
	No	5.7	5
Animal by-products operator	Yes	54.6	48
	No	45.5	40
Frequency of removal of animal origin by-products	Daily	69.3	61
	Weekly	20.5	18
	Fortnightly	0.0	0
	Another	9.1	8
Production of meat products	Yes	72.7	64
	No	27.3	24

Checklist analysis

With the checklist it was possible to evaluate the hygiene practices of the meat handlers and the hygiene and maintenance of the facilities. The mean “visual inspection” score among butcher shops was 64.0%, with a minimum of 26.0% and a maximum of 93.0% (Table 4).

Table 4 Results of visual inspection of butcher shops (N=73)

	Positive observation	
Visual inspection checklist	**%**	**n**
Hygiene of the meat handlers		
Use of cap	12.3%	9
Use of apron or gown	97.3%	71
Use of waterproof footwear	67.1%	49
Cleanliness of working clothes	65.8%	48
Working clothes - recent dirt	56.2%	41
Working clothes - ingrained dirt	15.1%	11
Nails short and clean	90.4%	66
Meat and money handling by the same operator	86.3%	63
Hygiene and maintenance of the facilities		
Existence of independent cutting and deboning room	54.8%	40
Existence of pest control	91.8%	67
Existence of cold and hot water	82.2%	60
Existence of sink with non manual control; disposable paper towels	90.4%	66
Cutting tables composed by innocuous material	97.3%	71
Presence of wood	24.7%	18
Presence of rust	37.0%	27
Presence of mold	20.5%	15
Protection of lamps	75.3%	55
Presence of brooms, cleaning cloths or detergents	60.3%	44
Meat presentation: in the same window display	94.5%	69
physical separation	100.0%	73
Meat products display	95.9%	70
Cleanliness of the establishment floor	63.0%	46
recent dirt	50.7%	37
ingrained dirt	20.5%	15
Cleanliness of the counter and hooks	50.7%	37
recent dirt	97.3%	71

ingrained dirt	34.2%	25

Hygiene of the meat handlers

The visual inspection of butcher shops, as described in Table 4, revealed that only twelve percent (12.3%) of the meat handlers used protective caps and around two thirds of them (67.1%) wore waterproof footwear. About one-third (34.3%) of the handlers presented unclean working clothes with around eighty five percent (84.9%) of these with ingrained dirt, and a small number (2.7%) were not wearing an apron or a gown over their clothing. The vast majority (90.4%) of the meat handlers had short clean nails. However, most meat handlers (86.3%) manipulating meat and cash at the same time, without hand-washing between these activities.

Hygiene and maintenance of the facilities

As described in Table 4, most of the butcher shops (94.5%) exposed meat from different species in the same window display with physical separation and around ninety six percent (95.9%) had meat products in separate window displays. Sixty-three percent of the butcher shops, had clean floors and, similarly, half (50.7%) had clean counters and hooks. Pest control devices were present in 91.8% of the facilities visited. On the other hand, whereas many (82.2%) had a hot water supply, on about an tenth (9.6%) of the premises visited sinks were not present and/or did not have manual control and disposable paper towels. Almost all facilities (97.3%) had cutting surfaces composed of innocuous and washable material, however between twenty and forty percent had surfaces/instruments made of wood (24.7%) and/or work surfaces that exhibited rust (37.0%) or mold (20.5%). In spite of the European Regulations and Portuguese law, more than half of the butcher shops visited (60.3%) presented brooms, cleaning cloths or cleaning products in sight. Seventy five percent (75.3%) of the facilities had lamps with protection against cracking.

Discussion

Meat handlers can be responsible for cross contamination and improper handling procedures that may contaminated meat and meat products and lead to

foodborne diseases*(5)*. This study evaluated food safety knowledge and practices in butcher shops and the level of compliance with EU food law regarding the food handling facilities and the personal hygiene of butchers and meat handlers in Central Portugal.

The social demographic data show that the profession of butcher is traditionally a job carried out by males, without advanced levels of formal education, but generally by people who have considerable years of experience. Previous reports established that despite their experience, people can adopt incorrect practices resulting from lack of formal education and the repetitive tasks associated with the job *(42, 4, 44)*. This profile can be associated with the errors demonstrated by the “Knowledge” and “Practice” scores (68.0%). This lack of knowledge and technically qualified personnel may translate into a greater risk for foodborne diseases, as observed in similar studies *(7)*.

Although the majority of the handlers declared they had training in GPFI and/or WSH during the last year, and all indicated that HACCP has been implemented, this did not seem to translate into high “Knowledge” and “Practice" evaluation scores. It would be expected that food safety training increased knowledge and improved hygiene practices. However, the evaluation of the checklists and questionnaires used in this study revealed several inappropriate food handling practices. Despite the operators’ training in basic concepts like HACCP or simple rules to reduce bacterial contamination (including use of different workbenches, mincers and knives for poultry versus beef or pork) compliance with the food safety law was inconsistent in the meat handing premises, in the present investigation. It is well known that the potential for foodborne illnesses can increase due to improper hygiene and handling practices involving food workers *(38, 33)*. More than 30.0% of the respondents reported the use of the same knife when preparing meat of different animal species. More than 60.0% affirmed that they used the same mincer for different species of meat and only around 31.0% said that they used different working surfaces to cut different species of meat. Surprisingly, 72.7% of the respondents did not separate different types of fresh meat and/or meat products into different bags at the moment of sale as required under EU regulations. More than representing examples of illegal

noncompliance *(9)*, these practices represent an increased risk of cross contamination*(3, 25, 33).*Our present findings indicate deficient hygienic conditions that can lead to an increased risk of foodborne pathogens transmitted through contaminated meats. Similar results were obtained in other studies, showing that training and HACCP implementation are often undertaken only to meet perceived statutory or inspection requirements and depend on the human resources knowledge and motivation *(27, 42, 4, 44, 17)*. In the case of the “Knowledge” and “Practice” evaluation, the mean score obtained was 68.0%, indicating, that the training that almost all participants obtained in the previous year or two failed to ensure the necessary or desired efficacy, as likewise reported in some other recent studies *(42, 17, 4, 44, 27)*.

According to the Commission Regulation (EC) No 1069/2009, operators that generate animal by-products or derived products, need an animal by-products operator and adequate containers in order to eliminate residues and segregate derived products not intended for human consumption, since they are a potential source of risks to public and animal health *(11, 16)*. In response to this risk control, the majority of the respondents affirmed that they immediately removed meat portions with clear or evident signs of damage. However, a small number (6.0%) of the establishments did not have an appropriate container and only around half of the butcher shops had an animal by-products operator. Despite this, 69.3% of the respondents said that theyremoved these residues every day. These data are inconsistent with the information provided, since 73.9% declared they had the support of an external company for the implementation and verification of HACCP. As a potential hazard, production and handling of animal by-products should be addressed in an establishment’s HACCP plan and proper controls should be implemented to address potential hazards. In spite of these irregularities, 72.7% of the butcher shops produced meat products. The data illustrate that lack of proper implementation of pre-requisite programs is a major hurdle to full implementation of the HACCP system. These data are consistent with earlier reports*(41, 36, 42, 17, 4, 44, 47, 48, 49)*.

Raw meat presents a health risk from the microbiological perspective *(7)* and, as a consequence, butcher shops pose an inherent risk due to the nature of

their products. In our study, more than 15.0% of the handlers wore clothing with ingrained dirt. As clothes represent a risk cause for cross-contamination, maintaining sanitary, clean personal protective equipment is critical to minimize microbial contamination *(6)*. However, in some cases we observed a lack of concern with personal hygiene and protective clothes. Unexpectedly, 86.3% of the meat handlers manipulated meat and cash without washing hands between activities. These results show, a failure to comply with the established rules of foodstuffs manipulation and, in some cases, the limited knowledge compliance with procedures among staff, as referred to in other studies *(4, 44, 7, 17)*.

In accordance with applicable law *(9)*, the majority of the facilities had pest control devices. This is extremely important in the control of the zoonotic transfer of pathogens from animals to foodstuffs *(29, 6)*. However, cats were seen in some of the facilities, in contradiction with the Portuguese law *(9)*.

Some of the butcher shops did not have a supply of hot water, and some did not have sinks with non-manual control and disposable paper towels affecting hand-washing practices and personal hygiene. Proper hand-washing and sanitizing of hands plays a critical role in food safety *(32, 37, 1, 34, 46)*. The complete procedure includes meticulous hand washing with hot water and efficient hand drying with paper towels; this contributes to a reduction in the risk of common infections and cross-contamination *(27, 2, 24, 45)*. The cleanliness and maintenance of the facilities are also extremely important to prevent cross-contamination and subsequently to maintain the safety and quality of meat *(18, 31)*. However, only half of the butcher shops visited had clean counters and hooks and more than 20.0% presented ingrained dirt on the floor. Portuguese law defines that the floor must be cleaned daily and all HACCP plans should include a cleaning and sanitization plan. Here also there was a lack of compliance with the law. Almost all of the facilities visited had cutting surfaces composed of innocuous and washable materials according to the Portuguese law *(9)*. However, between twenty (20.0%) and forty percent (40.0%) had surfaces or instruments composed of wood, or contaminated by rust or mold. This type of the material can promote or minimize the adherence of microbes to the surface *(43, 30, 31)*. In spite of the fact that wood was used in the past as a traditional material in the food industry,

nowadays polyethylene terephthalate (PET) is largely used and recommended due to its resistance and non-porosity *(23)*. More frequently than surfaces or instruments composed of wood, rust was found in the facilities. Moreover, rust is equally dangerous; this is a chemical contaminant needs to be controlled *(22)*. Fungi produce mycotoxins that can cause adverse health effects in humans like genotoxicity, cancer, kidney damage, gastric and reproductive disorders and suppression of the immune system *(19)*. More than this, fungi are responsible by the dissemination of spores, which are extremely resistant and dangerous for public health *(39)*.

European Regulations and Portuguese Law *(9)* are alert to physical and chemical hazards associated to the cleaning of the facilities. However, a high percentage of the butcher shops visited had brooms, cleaning cloths or detergents and disinfectants in sight, indicating storage practices that were not in compliance with the law. In order to control physical hazards, it is mandatory that all lamps be protected against cracking *(9)*. Nevertheless, some lamps in the butcher shops in this study were not protected, in contradiction with the implementation of HACCP. In several butcher shops some of the meat handlers were observed eating in the production area of the facilities, disregarding what most certainly was taught in GPFI training, and in clear contradiction to EU and Portuguese food law. Once again, it revealed that no critical point analysis was carried out. As mentioned above, similar difficulties were described in previous studies *(4, 44, 47, 48, 49)*.

An effort needs to be placed on the role prerequisite programs play in supporting the HACCP system. Once this relationship has been established, facilities can begin fully implementing prerequisite programs to address the HACCP implementation deficiencies observed in this study.

It is important to promote operative and continuing GPFI training for food and particularly meat handlers in Portugal. This plan should focus on raising the handlers' awareness of the implications that a disregard for the food safety guidelines has on public health and should be conducted or overseen by a national authority. Compliance with legal obligations determined by PACE is critical to the reduction of cross contamination and subsequent foodborne diseases. Meat consumption is projected to increase over the next 15 years

around the world *(19)*, particularly in Europe. Regarding this, we recommend that the appropriate authorities promote enforcement of the application of the HACCP principles, through more frequent official inspections to enforce food safety laws. It is also vital to evaluate the training impact and the general hygiene requirements and procedures as part of these proposed inspections. In addition, it would be important to educate people better concerning appropriate handling of meat and the importance of these practices in public health. We emphasize that these proposals aim to encourage a significant improvement of the safety and quality of meat and meat products currently marketed in Portuguese butcher shops upon which consumers rely. The effective compliance with the European and Portuguese law will consequently be crucial to improving the competitiveness of small retail meat businesses.

Conflict of interests

We declare no competing interests.

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References

1. Abd-Elaleem, R., W.M.K. Bark, W.A. Hazzah, and O. Nasreldin. 2014. Assessment of the personal hygiene and the bacteriological quality of butchers' hands in some abattoirs in Alexandria, Egypt. Food Control. 41: 147-150.

2. Allwood, P. B., T. Jenkins, C. Paulus, L., Johnson and C. W. Hedberg. 2004. Hand washing compliance among retail food establishment workers in Minnesota. Journal of Food Protection. 67: 2825-2828.

3. AU. Healthy Living and Environment. Australian Government. 2008. Available at:

http://www.pittwater.nsw.gov.au/__data/assets/pdf_file/0005/52484/Fact_Sheet_Cross_Contamination.pdf Accessed 07.06.2016.

4. Ball, B., A. Wilcock, and M. Aung. 2009. Factors influencing workers to follow food safety management systems in meat plants in Ontario, Canada. International Journal of Environmental Health Research. 19: 201-218.

5. Campos, A. K. C., Â. M. S. Cardonha, L. B. G. Pinheiro, N. R. Ferreira, P. R. M. d. Azevedo, and T. L. M. Stamford. 2009. Assessment of personal hygiene and practices of food handlers in municipal public schools of Natal, Brazil. Food Control. 20: 807-810.

6. CEHOG. 2012. Safe Food Handling for bucters. Crown Copyright.

7. De Boeck, E., L. Jacxsens, M. Bollaerts, M. Uyttendaele and P. Vlerick. 2016. Interplay between food safety climate, food safety management system and microbiological hygiene in farm butcheries and affiliated butcher shops. Food Control. 65: 78-91.

8. DGAV. 2013. Plano de Aprovação e Controlo dos Estavelecimentos (PACE). Plano PNCPI 2012-2014, Direcção Geral de Alimentação e Veterinária, Ministério da Agricultura e do Mar.

9. DL. 2008. Diário da República, 1.ª série N.º 206 - 23 de Outubro de 2008. Diário da República.

10. DL. 2006. Diário da Repúlica, Decreto de Lei 1.ª série N.º 146 Dec-lei 147/2006 31 Julho 2006. Diário da Républica.

11. EC. 2009. Commission regulation (EC) Nº 1069/2009 health rules as regards animal by-products and derived products not intended for human consumption. Official Journal of the European Union, 1-33.

12. EC. 2005. Commission regulation (EC) Nº 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Official Journal of the European Union, 1-26.

13. EC. 2004a. Regulamento (CE) N.º 852/2004 do Parlamento Europeu e do Conselho, de 29 de Abril de 2004, relativo à higiene dos géneros alimentícios . Jornal Oficial da União Europeia, 3-21.

14. EC. 2004b. Regulamento (CE) N.º 853/2004 do Parlamento Europeu e do Conselho, de 29 de Abril de 2004 , que estabelece regras específicas de higiene aplicáveis aos géneros alimentícios de origem animal . Jornal Oficial da União Europeia, 22-82.

15. EFSA. 2015. EU summary report on zoonoses, zoonotic agents and food-borne outbreaks 2013. EFSA European Food Safety Authoruty, 162.

16. EHOA. 2011. Butchers' Booklet - Guidance on Food Safety and Labelling Requirements relating to Butchers' Counters. (Vol. 1). Environmental Health Officers' Association.

17. Ellis, J.D., S.W. Arendt, C.H. Strohbehn, J. Meyer and P. Paez. 2010. Varying influences of motivation factors on employees' likelihood to perform safe food handling practices because of demographic differences. J Food Prot. 73: 2065-2071.

18. FAO. 2016. Meat processing technology for small-to medium-scale producers. http://www.fao.org/docrep/010/ai407e/AI407E23.htm Accessed 07.11.2016.

19. FAO. 2015. FAO, Animal Production and Health. Agriculture and Consumer Protection Department. http://www.fao.org/ag/againfo/themes/es/meat/background.htm Accessed 03.09.2016.

20. FAO. 1998. Food quality and safety systems. A training manual on food hygiene and the hazard analysis and critical control point (HACCP) system. Rome.

21. FDA. 2009a. FDA Food Code 2009: Annex 4 - Management of Food Safety Practices - Achieving Active Managerial Control of Foodborne Illness Risk Factors. Silver Spring: U.S. Food and Drug Administration. Silver Spring.

22. FDA. 2009b FDA Food Code 2009: Annex 3 - Public Health Reasons / Administrative Guidelines - Chapter 3, Food. Edited by U.S. Food and Drug Administration. Silver Spring.

23. Fink, R., S.M. Filip, M. Oder and M. Jevs̆nik 2013. Microbial pathogens and strategies for combating them: science, technology and education. Formatex.

24. Fry, A.M., C.R. Braden, P.M. Griffin and J.M. Hughes. 2005. Principles and practice of infectious diseases. Edited by J. E. Bennett, & R. Dolin (Eds.) G. L. Mandell. New York: Elsevier, Churchill Livingston.

25. FSA. 2013. Food Hygiene ~ A Guide for Businesses, (Vol. 1). Food Standards Agency.

26. Giusti, M.D., D. Medici, D. Tufi, C., Marzuillo and A. Boccia. 2007. Epidemiology of emerging foodborne pathogens. Italian Journal of Public Health. 4: 24-31.

27. Gomes-Neves E., C.S. Cardoso, A.C. Araújo, and J.M.C. Costa. 2011. Meat handlers training in Portugal: A survey on knowledge and practice. Food Control. 22: 501-507.

28. Gomes-Neves, E., A.C. Araújo, E. Ramos, and C.S. Cardoso. 2007. Food handling: Comparative analysis of general knowledge and practice in three relevant groups in Portugal. Food Control. 18: 707-712.

29. Hoorfar, J. 2014. Global safety of fresh produce: A handbook of best-practice examples, innovative commercial solutions and case studies. Edited by Technology and Nutrition Woodhead Publishing Series in Food Science. Woodhead Publishing.

30. Hori, K., and S. Matsumoto. 2010. Bacterial adhesion: From mechanisms to control. Biochemical Engineering Journal. 48: 424-434.

31. Ismaïl, R., F. Aviat, V. Michel, I. Le Bayon, P. Gay-Perret, M. Kutnik and M. Fédérighi. 2013. Methods for Recovering Microorganisms from Solid Surfaces

Used in the Food Industry: A Review of the Literature. Int J Environ Res Public Health. 10: 6169-6183.

32. Jensen, D.A., M.D. Danyluk, L.J. Harris and D.W. Schaffner. 2015. Quantifying the effect of hand wash duration, soap use, ground beef debris, and drying methods on the removal of Enterobacter aerogenes on hands. J Food Prot. 78: 685-690.

33. Jianu, C. and C. Chis. 2012. Study on the hygiene knowledge of food handlers working in small and medium-sized companies in western Romania. Food Control. 26: 151-156.

34. Jumaa, P.A. 2005. Hand hygene: simple and complex. International Journal of Infectious Diseases. 9: 3-14.

35. Legnani P., E. Leoni, M. Berveglieri, G. Mirolo and N. Alvaro. 2004. Hygienic control of mass catering establishments, microbiolohical monitoring of food and equipment. Food Control. 15: 205-211.

36. Luning, P.A., K. Kirezieva, G. Hagelaar, J. Rovira, M. Uyttendaele and L. Jacxsens. 2015. Performance assessment of food safety management systems in animalbased food companies in view of their context characteristics: a European study. Food Control. 49: 11-22.

37. Maughan, C., Iv. E. Chambers, S. Godwin, D. Chambers, S. Cates and K. Koppel. 2016. Food Handling Behaviors Observed in Consumers When Cooking Poultry and Eggs. J Food Prot. 79: 970-977.

38. McIntyre, L., L. Vallaster, L. Wilcott, S.B. Henderson and T. Kosatsky. 2013. Evaluation of food safety knowledge, attitudes and self-reported hand washing practices in FOODSAFE trained and untrained food handlers in British Columbia, Canada. Food Control. 30: 150-156.

39. Mohr, T.B., V.K. Juneja, H.H. Thippareddi, D.W. Schaffner, P.A. Bronstein, M. Silverman and L.V. Jr Cook. 2015. Assessing the Performance of Clostridium

perfringens Cooling Models for Cooked, Uncured Meat and Poultry Products. J Food Prot. 78: 1512-1526.

40. Nederhof, A.J. 1985. Methods of coping with social desirability bias: a review. European Journal of Social Psychology. 15: 263-280.

41. Oses, S.M. P.A. Luning, L. Jacxsens, S. Santillana, I. Jaime and J. Rovira. 2012. Food safety management system performance in the lamb chain. Food Control. 25: 493-500.

42. Ramalho, V., A. Pinto de Moura and L.M. Cunha. 2015. Why do small business butcher shops fail to fully implement HACCP? Food Control. 49: 85-91.

43. Raspor, P., and M. Jevšnik. 2008. Good nutritional practice from producer to consumer. Critical Reviews in Food Science and Nutrition. 48: 276-292.

44. Rasport, P. 2008. Total food chain safety. Trends in Food Science and Technolog. 19: 405-412.

45. Shojaei, H., J. Shooshtaripoor and M. Amiri. 2006. Efficacy of simple hand-washing in reduction of microbial hand contamination of Iranian food handlers. Food Research International. 39: 525-529.

46. Soriano, J.M., I. Prieto, J.C. Moltó and J. Mañes. 2005. A review of the application of the hazard analysis and critical control point system to salads served in the restaurant of Valencia University. International Journal of Food Science and Technology. 40: 333-336.

47. Taylor, E. 2001. HACCP in small companies: benefit or burden? Food Control. 12: 217-222.

48. Taylor, E., and K. Kane. 2005. Reducing the burden of HACCP on SMEs. Food Control. 16: 883-839.

49. Violaris, Y., O. Bridges, and J. Bridges. 2008. Small businesses - big risks: current status and future direction of HACCP in Cyprus. Food Control. 19: 439-448.

SECTION IV

DISCUSSION

IV. DISCUSSION

Meat, which imbalance?

Meat is,probably, one of the most controversialfoods in scientific literature and in social media. Recently, IARC/WHO declaration referring meat products as grade I carcinogens, re-open the controversy (WHO 2015g).The imbalance between an unquestionable nutritional value with historical dimension of meat, the increase of the meat consumption world while with subsequent increased production and associated health and environmental problems.

Smil (2002), and other authors, affirmed that eating meat is part of our evolutionary heritage and on human evolution process, with implications in teeth, brain, bones and psychological development(Smil 2002, Leroy 2015).Also, religious issues have been largely associated with the consumption of meat but, as described below, there is no religion in the world that fully rejects the meat consumption.The Jewish based their choices on *Kâser* or Kosher principles, which defines beef, sheep and goat as sacred meat and pork, hare and rabbit as non sacred meat. *Kâser* dietary laws focused on the animal kingdom, allow the consumption of the muscles of animals, but define the prohibition of blood and any mixing of milk and meat(Haris 1989, Regenstein 2003).For Catholics, the year is divided in fat days or “the meat” and prayers days or slim (Lent, Advent, Fridays and Saturdays) that prohibited meat and animal fat consumption (Conteras 2007). Muslim population divides food into *h’alâl* (permitted) and *h’arâm* (prohibited) food prohibiting pork meat consumption(Haris 1989, Regenstein 2003). Traditionally, Hindus do not eat any kind of meat (Conteras 2007). In addition to particular religion restrictions, there is an environmental pressure caused by meat supply chains and particularly by beef chain (Walker 2005).

According to our analysis from a selected bibliography it is possible to conclude that eating meat is favorable for human health. However, some areas of concern remain: health and metabolic problems associated to high intake of meat, food borne diseases associated with meat contamination, or even, environmental

impact caused by meat overproduction (OMS/FAO 2003, WHO 2015f, Kouvari 2015, WHO 2015g, WHO 2015g).

In spite that for many thousands of years, mankind has lived in close proximity with numerous animal species, which have provided food and shelter in exchange for their domestic use, today the meat consumption is extraordinary different. With the increase of world population, a global increase of the meat production sector is clearly seen in Figure 3. Environmental impacthas aninfluence in three dimensions – climate change, consumption of natural resources and environmental pollution. Meat production has a greatest environmental impact due to the inefficiency of animals in converting feed to meat (Djekic 2015).The environmental aspects with major impact are discharge of wastewater and solid wasteand consumption of water and energy (IPPC 2006, IFC 2007). In terms of significant impact, the major problems are emissions ofmethane, nitrous oxide and carbon dioxide from manure, potential acidification and eutrophication and the use of natural resources, namely water and energy (Dalgaard 2007, Reckmann 2012).In EU, permanent grasslands may represent a sink of 11.5 ± 69.0 million tonnes CO_2-eq per year, or 3% ± 18% of greenhouse gas (GHG) emissions just from the ruminant sector (Gerber 2013).

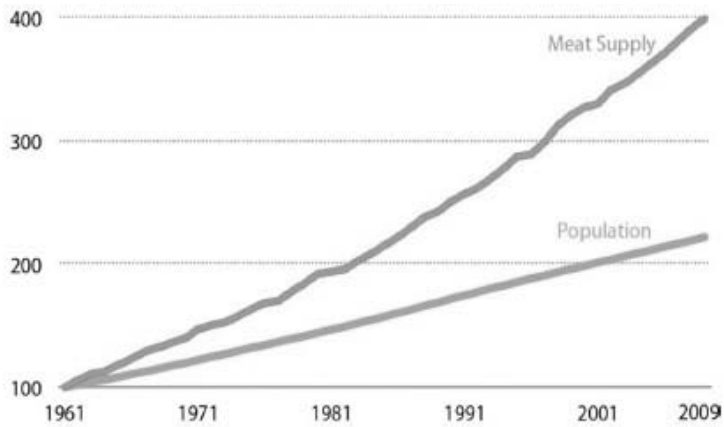

Figure 3. Growth of population and meat supply, indexed 1961=100(UN 2012, FAO 2012).

More than a negative environmental impact,unfortunately the intensive meat production had, in the last years, a negative impact in the quality of meat produced and subsequently, in human health. The abusive usage of the veterinary drugs

(Amadori 2016)and the needs to produce more with less,have contributed, during the last years, for the decreased of the meat quality(Thornton 2010).

On the other hand, we are currently witnessing dangerous increase of meat consumption.In Portugal in 2014, there were consumed 108.1Kg *per capita* (INE 2015), 43.2Kg more than in the same period in EU (OCDE 2015) and 75.1Kg/*per capita* more than the FAO recommendation. The scale of the social and health problem due to excessive consumption of meat calls for the mobilization of all efforts to reduce it. The indiscriminate meat consumption now is recognized as an epidemic health problem (Matsuoka 2013, WHO 2015d).

This indiscriminate consumption is having an increasing impact on environmental and on human health. Different authors haveshown that if the animal welfare is compromised, there are significant negative human health consequences promoted by the environmental degradation, the indiscriminate use of veterinary drugs and the consequences of intensification (Matsuoka 2013, Goldberg 2016).A responsible consumption of meat willpromote an environmental stability and maintenance of the meat level in human diet in the future(Goldberg 2016). Astrongly modification of the industrial production with a reduction of the antibiotic use (Goldberg 2016), probiotic consumption and with an increase of the animal welfare and animal respect by the humans is crucial(Matsuoka 2013). More than these direct impacts in human health, the expansion of livestock production is a direct cause of deforestation, particularly in Latin America(Matsuoka 2013).

Meat as food borne source of infection was an important question, during our work.Due to it endogenous characteristics, meat is a food borne source of infection but, with the significant increase of meat production and consumption globally, this problem is moresignificant. During all meat chain steps, handlers need tofulfill the HACCP and GMP principles (Gomes-Neves 2012, Ramalho 2015) and theEU food law. As consequence of the increase of the production, and the food borne contamination risk, is crucial to develop new first line diagnostic tests, to control biological and chemical hazards. These tests should be rapid, sensitive, specific and economicto promote an efficient control of the meat chain and to control the level of the food borne diseases associated to the meat consumption(Fisher 2007).

FiveRiskstest development.

After careful consideration about meat and meat market, prevailed the meat market requirements with the development of a new first line diagnostic test. These new diagnostic tests, at the same time, have to be rapid, sensitive and economic. FiveRisks is more than a simple diagnostic test, is until this moment and to our knowledge, the unique test that detects, at the same time, three foodborne pathogens (*Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes)* and two process hygiene indicators(*Staphylococcus* coagulase positive and *Escherichia coli)*. These pathogens were chosen because, according to the recent EFSA and OMS reports, are transversal of different type of foods and in chain production(WHO 2004, EFSA 2007, WHO 2015d, EFSA 2015a, EFSA s.d.)

A key step in the development of this assaywas DNA extraction. An enzymatic method on the first lysing cell step, was selected because lysozymeis used to disrupt Gram positive cells by hydrolyze the b-1,4 glycoside linkages between GlcNAc and MurNAc of the glycan backbone (O. 2008).

The PCR optimization was difficult due to some technical limitations. The accuracy of mPCR was evaluated at different levels. Initially, DNA of each reference strain was used witha mix containing all 5 pairs of primers. Any nonspecific band was observed. On the second step, DNA from all bacteria was mixed in the same reaction mix and*S.* coagulase positive gene was not amplified. As referred previously, several experiments were performed to improve mPCR. In this attempt, several reaction conditions were performed, namely the number of extension cycles, primers and DNA concentration, primer melting temperatures etc. Results were not satisfactory in previously defined conditions. So, a combination of *S.* coagulase positive DNA and *Campylobacter* spp. DNA was proposed in a single reaction with a mix of five pairs of primers; in a separated set, *E. coli*, *L. monocytogenes* and *Salmonella* spp. DNA was amplified with the mix of five pairs of primers. In these conditions, multiplex PCR amplification was successful. There could be a multiplex PCR saturation, because despite the attempt to normalize the amount of sample in reaction (1CFU), and the several experiments that were performed to improve mPCR, it was possible to see that the

intensity of the bands in agarose gel was different. The genes of *Salmonella* spp. and *Listeria monocytogenes*, could be saturating the thermostable DNA polymerase. In the case of mRT-PCR itwas not occurringprobably, due to the higher sensitivity of the method.

Regarding sensitivity, a limited dilution approach was used, as mentioned before. Starting from DNA extracted from 1CFU, we performed several dilutions and submitted these to PCR. A decrease in the amount of PCR product was observed with the increase in dilutions. The limit was reveled when a further dilution did not generate any PCR product. Then, DNA from each pathogen was quantified by using NanoDrop™ and Quant-iT™ PicoGreen®. Surprisingly, it was difficult to quantify the amount of DNA even in the non-diluted samples with either technique. For this reason it was not possible to determine the amount of DNA present in the dilutions to infer the sensitivity of each mPCR. This may be explained by the sensitivity of NanoDrop™ apparatus (up to 2ng/µl), and the Quant-iT™ PicoGreen® approach, which ranges from 50 pg to 2 µg. The PCR sensitivity, depending on the target and other factors, reaches lower values, but this is not true for the quantification techniques used. These facts justify the positive PCR amplification from all the samples even if the DNA quantification did not work.

After PCR optimization, it was possible to develop the first optimization step using RT-PCR.A plasmid based approach was made to evaluate with higher accuracy, the sensitivity of the mRT-PCR reactions. Briefly, the five plasmids were synthetized in a *pUC57* vector. To eliminate intra-assay variability, all plasmids were analyzed in replicates. It is possible to see in Chapter II, there are universal fluctuations that do not produce obvious distortions in the reporter signal, but they do affect the precision of replicates. These fluctuations in the fluorescent signal, are due to the refluxing that occurs into the wells, the small air bubbles that are creating into the wells and because of the pressure that the steam exert on the plastic seal, that promote a change shape slightly during the reaction time. A statistic analysis of FiveRisks was obtained R^2 minimum = 0.983 and the R^2 maximum = 0.999.

Another issue is that was designeda multiplex RT-PCR and not a singleplex reaction because this increased throughput, reduced sample quantity needed (DNA) and minimize the pipet precision errors. This mRT-PCR was developed with TaqMan® chemistry assay because, despite being more expensive than DNA binding assays, the hydrolysis of the probes ensures that only specific amplicons are measured(Postollec 2011).

During all steps, normalization methods were used to eliminate experimental inconsistencies and neutralize the effects of the variability. This normalization was performed using the normalizing of sample quantity. In all samples, the DNA isolation started with the same amount of sample. In this case, weknow that it would have been important normalizing the DNA quantity and were did it in the sensitivity and sensibility assays validation, with all successive validation matrices. To confirm this normalization during the validation period,most of the analysis were made with replicates and all present results, were tested more than once. All of these procedures allowed creating a high efficient, sensitive and reproducible experiment.

In the case of the spiked samples, it is important to refer that the sensitivity of the assay depends, particularly, of the food matrix. The extraction method is the same in all foodstuffs, but the result is different. It occurs because all of these foodstuffs have some proteinase inhibitors, in the case of meat samples there are a lot of enzymes and heme groups; in dairy products, the lactose and other sugars promote this inhibition and in case of vegetables, polyphenols and sugars are largely described as responsible for the reaction inhibition (Schrader 2012). Some authors described the qPCR analysis without enrichment of food samples, but the limit dilution of these assays is less that with enrichment, were in the range of 10^2 - 10^3 CFU/mL (or /g) (N. E.-Z. Hierro 2006, N. E.-Z. Hierro 2007, Takahashi 2009). In order to meet the microbiological criteria required by European Legislation for foodstuffs, it is sometimes necessary to associate qPCR with an enrichment step of a few hours (Postollec 2011). Considering that the minor limit detection is 10 - 10^3 CFU/g, this assay does not require the incubation step but in order to respect microbiological criteria required by European Legislation, incubation is suggested.

All procedures were made based on EN ISO 16140 guidelines for the validation of alternative methods and they included in validation assays, a positive control constituted by commercial reference DNA, a non-template control (blank) with water in place of the sample and in case of microbiological procedure, a control was used for environmental contamination during handling.

Despite of the excellent FiveRisks performance, positive cases must be oriented for confirmation of the presence of the viable pathogens, at the national reference laboratory. A regulatory approval is required according to ISO 22174:2005- Microbiology of food and animal feeding stuffs -- Polymerase chain reaction (PCR) for the detection of food borne pathogens. This standard gives the general requirements for the *in vitro* amplification of nucleic acid sequences and is applicable to foodstuffs and isolates obtained from foodstuffs for foodborne pathogens using the PCR (ISO 2014). The main objective of this standard is ensure that comparable and reproducible results are obtained in different laboratories.

Butcher shops analysis: a challenge and a necessity.

In a global perspective, the main objective of the Chapter III was evaluating a potential market for FiveRisks.

Meat handlers are extremely important in meat market but, on the other hand, can be responsible by cross-contamination motivated by improper handling (Campos 2009, Kibret 2012, Muñoz 2013). As a consequence, there are a lot of foodborne diseases that could be avoided with correct GPFI and HACCP plan implementation and accomplishment. In this Chapter III were evaluated the food safety knowledge and practices in butcher shops and the level of EU food law compliance regarding the facilities and personal hygiene of the handlers.

Not surprisingly, the social demographic evaluation shows that this is a traditional job. More than 70% of the respondents were men with low schooling; just 4.5% had higher education. We think that this fact had an important impact in our results, namely in hygiene and safety status, as described in other similar studies. De Boeck (2016) compare in a recent study, affiliated butcher shops and

farm butcheries and conclude that food safety climate was scored higher by affiliated butcher shops than by farm butcheries; more than this, he concludes that affiliated butcher shops have a higher microbiological hygiene and safety status. This study shows that the lack of knowledge and technically qualified personal may translate into a greater risk for foodborne diseases (De Boeck 2016). Despite of 93.2% of respondents had more than 5 considerable years of experiencethe “knowledge” and “practice” score obtained was 68 %. This result demonstrates the bad manufacturing practices identified. Recent studies corroborate these data and demonstrate that despite of the experience and the professional training, workers can adopt incorrect practices promoted by the low schooling (Ramalho 2015, Ball 2009).

All participants were requested about training. Ninety-seven point seven percent of the employees affirm that had training in GPFI and the same percentage affirm that had training in WSH. The majority affirms that had training on the last two years.In view of these data, a good score was expected but, surprisingly the mean scores for “knowledge” and “practice” questions was 68 %, with 43 % as minimum and 93 % as maximum. Despite of the regulatory efforts that have been undertaken in order to increase employees training, and subsequently promote a reduction in foodborne episodes, these results demonstrate that the training was ineffective. Similar results were obtained in other studies in butcher shops, meat plants and slaughterhouses(Ball 2009, Ball 2010, Ellis 2010, Gomes-Neves E. 2011, Ramalho 2015).

Three “knowledge” questions were constructed in order to understand if operators had basic notions of foodborne diseases and HACCP. The results obtained confirm the score presented above. Once again, a certain training ineffectiveness was observed because would be expected that food safety training increase knowledge. Just 23.9% of the respondents identified the risk groups for foodborne diseases. The majority of the employees identified correctly the common signs of the foodborne infection. However, less than half of the respondents correctly identified the HACCP definition. All butcher shops visited claimed they had HACCP and a hygienic plan implemented and 73.9% had a subcontracted company to help in this implementation. Unfortunately, as we will

discuss below, a number of prerequisites are not being complied. This is a common situation reported in other scientific studies. Particularly in SMEs, HACCP implementation is often undertaken only to meet perceived statutory or inspection requirements and is very dependent of the human resources knowledge and motivation (E. Taylor 2001b, E. K. Taylor 2005, Violaris 2008, Ball 2009, Ellis 2010, Gomes-Neves E. 2011, Oses 2012, Ramalho 2015, Luning 2015).

Food safety training should improve hygiene practices (Rivas 1982, McCartan 1982, Park 2010, Adesokan 2015) but, despite of the majority of the participants in this study had this training, simple rules that promote the reduction of the cross-contamination were ignored. A few simple practices like the use of different mincers, knives or workbenches were not applied. Only 48.9% use different knives for handling meat from different animal species; 60.2% of the butcher shops use the same mincer and 52.3% use the same workbench to prepare meat from diverse animal species. This is extremely worrying if we take into account that poultry meat is one of the most important food borne source of infection in meat chain(CDC 2008, Suzuki 2009, Clark, M. 2016).In line with the last data, 72.7% of the respondents do not separate different types of fresh meat and/or meat products into different bags in the moment of sale. More than a non-compliance with the law (DL 2008) this represents a poor hygiene practice and an increased risk of cross-contamination (AU 2008, Jianu 2012, FSA 2013).

One of the focalregulatory requirements according to the Commission Regulation (EC) No 1069/2009 is the management of animal by-products or derived products. According this regulation, all operators that generate animal by-products need an adequate container and an operator in order to eliminate these residues and derived products not intended for human consumption, since they are a potential source of risks to public and animal health (EC 2009, EHOA 2011). Five point seven percent of the butcher shops visited affirms that do not have an appropriate container and 45.5% do not have an operator. During the interviews, a lot of respondents affirm that provide animal by-products to costumers, being this situation included in the applicable law, however this can not be the only elimination process of the butcher shop. This fact shows some recklessness towards environmental impact and public health. However, when employees were

asked to explain what they do when one meat portion shows clear signs of damage, more than 84 % affirms that removed this immediately. No matter whether they have an appropriate container or not, 69.3 % affirmed that these residues were collected daily. Failure to comply with a legal duty to process correctly animal by-products has shown also the inefficiency of the HACCP system implemented in all establishments.

Despite of the non-accomplish of a fewHACCP principles, 72.7% of the butcher shops visited produce meat products. This production should be done under hygienic conditions and in accordance with GPFI and HACCP principles. Unfortunately, in this study it was confirmed the lack of several prerequisites as cutting room or animal by-products container.

More than a “knowledge” and “practice” evaluation a visual inspection of hygiene of the facilities and the operators and the maintenance of the butcher shops has been made. One more time, despite of the food safety training of the employees, the mean score obtained in this section was 64 % with a minimum of 26 % and a maximum of 93 %. As will be discussed further below, poor handling practices and deficient hygienic conditions can be promoting food borne illnesses (Jianu 2012, McIntyre 2013).Raw meat is a product with microbiological risks (De Boeck 2016) as a consequence, butcher shops have microbiological risk associated due to the nature of the products that they deliver. Under these circumstances,it is important inferthe hygienic conditions of the operators and the facilities.

Personal protective equipment has been evaluated. Contrarily to what is disposed in the law only 12.3% of the employees used a cap, 67.1% used waterproof footwear and 2.7% were not usingapron. As described in other studies, the limited knowledge of the operators can be related to the little concern with personal protection and the lack of awareness of the associated risks(Ball 2009, Ellis 2010, De Boeck 2016). More than a risk for operators, the lack of hygiene represents an increased probability of cross-contamination. In terms of cleaning, 65.8% of the employees were wearing clean working clothes but 15.1% presented ingrained dirt. This is a relevant value as we consider that clothes can be one of the main responsible for the cross-contamination (CEHOG 2012).

As far as butcher shops facilities were concerned, 91.8% had pest control devices. This is crucial to control the zoonotic transfer of pathogens from animals to foodstuffs (CEHOG 2012, Hoorfar 2014)and it is a legal requirement (DL 2008). Nevertheless, there have been observed cats in some butcher shops. This shows, one more time, the operators thoughtlessness and the ineffectiveness of the HACCP system implemented.

According to recent studies, one of the critical roles in food safety is the correct hand sanitizing (Jumaa 2005, Soriano 2005, Abd-Elaleem 2014, D. D. Jensen 2015, Maughan 2016).This procedure contributes to reduce the risk of common infections and cross-contaminations (Allwood 2004, Fry 2005, Shojaei 2006, Gomes-Neves E. 2011). A correct hands sanitizing includes a meticulous hand washing with hot water and an efficient drying hand with paper towels. All of this is put at risk because just 82.2% of the establishments had cold and hot water and around 10 % do not have sink with non-manual control and disposable paper towels.More positive results were obtained for nails cleansing, since over than 90% of the respondents had it short and clean. However, 86.3 % of the operators surveyed, manipulated at the same time meat and cash.

The physical risks control is one of the objectives of the HACCP principles, moreover, Portuguese law determined that all lamps need protection (DL 2008). Nevertheless, only 75.3% of the establishments visited fulfill this requirement. In order to control chemical risks, Portuguese law decline the use of brooms, cleaning cloths or detergents in the butcher shops during opening hours but (DL 2008) one more time, more than 60 % of the establishments visited had this objects present during the work, near to the meat. This represents other HACCP principles violation.

Recent reports and studies demonstrate that facilities cleanliness is also extremely important to prevent cross-contamination and subsequently to foster the sale of safety and quality meat (Ismaïl 2013, FAO 2016). As a reaction to these facts, the state of cleanliness of the floor, the counter and hooks was observed. Twenty point five percent of the butcher shops visited had ingrained dirt in the floor and 34.2% present ingrained dirt in counter and hooks. These results compromise, one more time, the hygiene of the working environment and consequently the

safety of meat. This demonstrates the non-compliance of the hygienic plan implemented reported as implemented in all the establishments. In order to evaluate the maintenance of the facilities, the rust and mold presence were checked. Thirty seven percent of the butcher shops visited had rust and more than 20 % had mold on the floor, on the walls or on the ceiling. According to FAO, fungi are responsible by the mycotoxins production; these toxins can cause adverse health human effects like genotoxicity, cancer, kidney damage, gastric and reproductive disorders and suppression of the immune system(FAO 2015a). In food chain, and particularly in meat chain, fungi are extremely dangerous because they are responsible by the spores' dissemination that are extremely resistant to traditional cleaning methods and develops due to the presence of high levels of humidity (Mohr 2015). Rusty is a chemical contaminant equally dangerous to human health and need to be removed (FDA 2009b). Again this represents a violation of the law.

Other important contamination focus was observed in cutting tables. According to recent data, the type of the surfaces' material is responsible by the promotion or the minimization of the microorganisms' adherence (Raspor 2008, Hori 2010, Ismaïl 2013). The recommended cutting surfaces material is polyethylene terephalate (PET) due to the resistance and non-porosity (Fink 2013). Wood was previously used but law is clear and disclaims the use of porosity materials. In this study,were observed 2.7% of the butcher shops with cutting tables with non-innocuous material.In 24.7% of the establishments was observed wood for example in cutting tables and instruments(knifesoraxes). Once again,HACCP prerequisites failed.

Meat exposure received special attention because all butcher shops sold fresh meat and meat products. Ninety five point nine percent of the establishments have a display counter for meat products thoroughly complying with the law.

Other worrisome data indicate that only 54.8% of the establishments have cutting room. This is relevant if we take into account that 72.7% of the butcher shops affirms that produce meat products. The European and Portuguese laws are clear: a cutting room is mandatory in these cases.

During the visits to the butcher shops, there was the opportunity to observe employees that during a break in the work, were eating and drinking on top of the cutting tables. It were also observed other lacks in the hygiene and law requirements, as the floor covered with cardboards, ceilings with holes, and toothbrushes on the sinks and even operators that were brushing the hair near to the workbench. All of these procedures have shown an absolutely lack of knowledge of the HACCP principles, GPFI and particularly the EU and Portuguese food law. In addition, they showed a completely unconscious to the foodborne associated risks.

All results analyzed above seem toshow that no critical point analysis was carried out. In these conditions it is absolutely impossible to implement an efficient HACCP system. Similar results have been described in SMEs (E. Taylor 2001b, E. K. Taylor 2005, Violaris 2008, Ball 2009).Nevertheless, in contrast with these studies, here, all establishments declare that they had an HACCP system implemented and a significant number of the participants have an external specialist or company to help in this implementation. These results shown a contradiction between what is reposted and may indicate the inefficiency of the enterprises responsible by the implementation and maintenance of the HACCP systems. This also points to the need of more efficient training and the effective official control of the facilities and practices in butcher shops.

In Portugal, a“handler card”is mandatory for people working in the meat retail businesses(DL 2006). To obtain this card, the professional must attend 15 hours of mandatory training in meat hygiene, food microbiology, handlers’ personal hygiene, working spaces and equipment hygiene, packaging of meat and meat products, hygiene of meat selling and delivery, food safety, HACCP and WSH(DL 2006, Gomes-Neves E. 2011).Despite of the majority of the employees affirm that had training on the last two years, serious deficiencies were observed during the butcher shops visits. The lack of awareness among meat handlers as to the foodborne risks associated to the bad manufacturing practices promote public health risks associated to the meat consumption.

In accordance with European requirements and Portuguese law, Portuguese national veterinary authority create PACE, a program that includes

different checklists applicable to the establishments that produce animal origin food. The checklists are intended to support control visits to specific facilities that produced, for example, meat products. The main objective of this official program in accordance with the food laws is promoting the GPFI and the reduction of the cross-contamination and subsequent foodborne diseases (DGAV 2013). Unfortunately, different items of the respective PACE checklist were not being complied in the butcher shops visited and this seems to demonstrate that the official inspections should be intensified.

According to FAO estimates, meat consumption will increase over the next 15 years (FAO 2015b) in all developed countries. As a consequence, the foodborne diseases can increase if appropriate action is not taken. It is not enough to affirmthat all butcher shops have anHACCPimplemented system; it is essential to comply with all the prerequisites. After a correct implementation, all butcher shops can work under GPFI conditions. To promote these improvements, Portuguese Authorities should carry out official inspections in order to verify the correct application of food safety laws.

In addition, particular attention should be paid to training impact in general hygiene requirements. In our visits, we observed that despite being a traditional job, meat handlers are willing to change in order to improving the competitiveness of thesmall retail meat businesses.

SECTION V

CONCLUSIONS AND PERSPECTIVES

V. CONCLUSIONS AND PERSPECTIVES

The main objectives of this work was study the impact of meat consumption in human health and develop a new diagnostic test, based on a Multiplex Real-Time PCR, to detect bacterial contamination.On the last step, was determinant analyzebutcher shops, their characteristics and the impact of the manufacturing practices in cross-contamination risks.

Despite of the meat consumption importance worldwide, this excessive growth in consumption increases the risk ofmetabolic and chronic diseases, including diabetes type II or cardiovascular diseases, cancer, and food borne diseases. To control this, is crucial develop a consumer education in order to respect the recommended nutritional intake of meat and meat products. In addition, is a strictly necessary promoting food safety practice in meat chain and in home kitchens to minimize risks related with cancer, chronic diseases and food borne illness.

Meat chain is critical for human health not only because of the health risks associated with an exaggerated consumption, but also due to the problems observed along the production chain. One of the most important meat chain steps are butcher shops. It was evaluated in this work and it was possibleevaluate if the guidelines and conditions to sale fresh meat and meat products, determining by the European regulations were being met, in Portuguese butcher shops (in Center of Portugal). It has been demonstrated that, some of these European guidelines are not delivering. Despite of more than 90 % of the meat handlers had training on the last two years, this training has not been effective because a lot of non-compliances were observed. The mean “knowledge” and “practice” score among operators was 68 % and the mean “visual inspection” score among butcher shops was 64 %. These non-compliances, put into question the safety and quality of the meat. In the future it will be important increase the sampling to another geographical area; on the other hand, it would be important to promote a debate with meat sector associations, and authorities, to sensitize people to the correct meat handling and the importance of these practices in public health.

These results justify a development of a new first line diagnostic test, fast and easy to use in order to control this meat chain step.FiveRisks, the main objective of this thesis, was developed with the goal of ensuring the meat safety and quality.

This new diagnostic test, will ableto detect, at the same time, three pathogens, *Salmonella* spp., *Campylobacter* spp. and *L. monocytogenes*, and two important hygiene indicators, *E. coli* and *S.* coagulase positive. With this new test, will be able to detect just in one work-day, in meat and meat products, all of these bacteria described above, with 100% of sensibility and a limit of detection of 10 CFU/g of meat for *Salmonella* spp., and *Escherichia coli*, 10^3CFU/gfor *Campylobacter* spp., 10^6CFU/g for *L. monocytogenes* and 10^4CFU/g meat for*S.*coagulase positive, without an enrichment step. According to these results, itwill be important develop in the future, new assays with an enrichment step. More than an enrichment step, it will be important develop a large-scale study with meat samples provided by the different steps in meat chain, to understand if there are cross-contamination between chain steps. It will be important to validate this method, according to ISO 22174:2004 and commercialize this, not only to meat market, but also to dairy products market.

The use in massive scale of this diagnostic test may have consequences in economic and social terms, improving the meat safety and quality and as a consequence improvingpublic health with the reduction of the foodborne diseases.

SECTION VI

APPENDIX

VI. Appendix I

Protocol to Detect DNA of *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter* spp., *Staphylococcus aureus* and *Escherichia coli* in foodstuffs

1st. Principle - Specific DNA fragments of the *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter* spp., *Staphylococcus aureus* and *Escherichia coli* genes are amplified by multiplex Real-Time PCR using five pairs of primers and five specific probes.

2nd. Samples preparation - Homogenize in a stomacher bag, 10g of any sample (the protocol were optimized to meat, cheese and green juice) with 90mL of Peptone Water, during 60'' at medium pressure. Separate 1mL of this homogenized and proceed according to the extraction protocol.

3rd. DNA Extraction - DNA extraction were optimized using QIAamp® DNA Mini and Blood Mini Handbook. Follow the protocol to isolation of genomic DNA from Gram-positive bacteria.

4th. Reagents and materials for the multiplex RT-PCR

a. Reagents for multiplex RT-PCR

i. Nuclease-free water
ii. RT-PCR mix - iQ™Multiplex Powermix (2X) (Bio rad)[‡]
iii. Primers and Probes at 300 µM
iv. RT-PCR Enzyme - iTaq™ DNA Polymerase (5 U/µL)[§]

b. Materials for multiplex RT-PCR

i. Microcentrifuge tubes
ii. Pipette tips, preferably aerosol barrier tips

[‡] The protocol were optimized using this mix, it can be replaced but is not guaranteed to maintain the sensitivity and specificity of the assay.

[§] The protocol were optimized using this Taq DNA Polymerase, it can be removed/replaced but is not guaranteed to maintain the sensitivity and specificity of the assay.

iii. Plates for thermal cycler

5th. Apparatus

a. Vortexer
b. Microcentrifuge
c. C1000 Touch™ Thermal Cycler

6th. multiplex RT-PCR protocol

a. 3’ at 95ºC; 30 cycles of 30’’ at 95ºC, 30’’ at 56ºC, 30’’ at 72ºC; 5’ at 72ºC.

SECTION VII

REFERENCES

VII. REFERENCES

Abd-Elaleem, R. Bark, W.M.K., Hazzah, W.A., Nasreldin, O. “Assessment of the personal hygiene and the bacteriological quality of butchers’ hands in some abattoirs in Alexandria, Egypt.” *Food Control* 41 (2014): 147-150.

Abdel-Hafeez, E.H., Kamal, A.M., Abdelgelil, N.H., Abdel-Fatah, M. “Parasites trasmitted to human by ingestion of different types of meat, el-minia city, el-minia governorate, Egypt.” *J Egypt Soc Parasitol* 45 (2015): 671-680.

Abitbol, M. “Speculation on posture, locomotion, energy consumption, and blood flow in early hominids.” *Gait & Posture* 3 (1995): 29-37.

Adesokan, H.K., Akinseye, V.O., Adesokan, G.A. “Food Safety Training Is Associated with Improved Knowledge and Behaviours among Foodservice Establishments' Workers.” *Int J Food Sci.* 2015 (2015): Published on line.

AG. 27 07 2015. https://www.eatforhealth.gov.au/food-essentials/five-food-groups/lean-meat-and-poultry-fish-eggs-tofu-nuts-and-seeds-and (accessed 06 08, 2016).

AHA. *The American Heart Association.* 08 2015. http://www.heart.org/HEARTORG/HealthyLiving/HealthyEating/Nutrition/The-American-Heart-Associations-Diet-and-Lifestyle-Recommendations_UCM_305855_Article.jsp#.VvAiU2SLRhA (accessed 03 21, 2016).

Alarcón, B., B. Vicedo, and R. Aznar. “PCR-based procedures for detection and quantification of Staphylococcus aureus and their application in food.” *Journal of Applied Microbiology* 100 (2006): 352-364.

Allerberger, F., Wagner, M. “Listeriosis: a resurgent food-borne infection.” *Clinical microbiology and infection* 16 (2010): 16-23.

Allwood, P. B., Jenkins, T., Paulus, C., Johnson, L., Hedberg, C. W. “Hand washing compliance among retail food establishment workers in Minnesota.” *Journal of Food Protection* 67 (2004): 2825-2828.

Amadori, M., Zanotti, C. “Immunoprophylaxis in intensive farming systems: the way forward.” *Vet Immunol Immunopathol.* 20 (2016): [Epub ahead of print].

Anderson, A.S., Key, T.J., Norat, T., Scoccianti, C., Cecchini, M., Berrino F., Boutron-Ruault, M-C., Espina, C., Leitzmann, M., Powers, H., Wiseman, M., Romieu, I. “European Code against Cancer 4th Edition: Obesity, body fatness and cancer.” *Cancer Epidemiology* 39S (2015): S34-S45.

Andreoli, P., Thijssen, J., Anthony, R., Vos, P. De Levita, W. Fast method for detecting micro-organisms in food samples. Ed. International patent WO 2004/106547 A2. April 2004.

Arean, U. *Nutrition and dental health.* Oxford: Oxford Medical Publications, 1993.

AU. *Healthy Living and Environment.* Australian Government. 07 2008. http://www.pittwater.nsw.gov.au/__data/assets/pdf_file/0005/52484/Fact_Sheet_Cross_Contamination.pdf (accessed 07 06, 2016).

Auger, C., Said, A., Nguyen, P.N., Chabert, P., Idris-Khodja, N., Schini-Kerth, V.B. “Potential of food and natural products to promote endothelial and vascular health.” *J Cardiovasc Pharmacol* 11 (2016): Epun ahead of print.

Aznar, R., Alarcón, B. “On the specificity pf PCR detection of Listeria monocytogenes in food: a comparison of published primers.” *Systematic and applied microbiology* 25 (2002): 109-119.

Balaban, N., Rasooly, A. “Staphylococcal enterotoxins.” *International Journal of Food Microbiology* 61 (2000): 1-10.

Ball, B., Wilcock, A., Aung, M. “Background factors affecting the implementation of food safety management systems.” *Food Protection Trends* 30 (2010): 78-86.

Ball, B., Wilcock, A., Aung, M. “Factors influencing workers to follow food safety management systems in meat plants in Ontario, Canada.” *International Journal of Environmental Health Research* 19 (2009).

Barbosa, J., Cruz, C., Martins, J., Silva, J.M., Neves, C., Alves, C., Ramos, F., Da Silveira, M.I.N. “Food poisoning by clenbuterol in Portugal.” *Food aditives and contaminants* 22 (2005): 563-566.

Baylis, C., Uyttendaele, M., Joosten, H., Davies, A. *The enterobacteriaceae and their significance to the food industry.* ILSI Report Series, Brussels: ILSI Europe Emerging Microbiological Issues Task Force, 2011.

Becker, K., Roth, R., Peters, G. “Rapid and specific detection of toxigenic Staphylococcus aureus: use of two multiplex PCR enzyme inmunoassays for amplification and hybridization of staphylococcal enterotoxin genes, and toxic shock syndrome toxin 1 gene.” *Journal of clinical microbiology* 36 (1998): 2548-2553.

Bennett, S., Walsh, K.A., Gould, L.H. “Foodborne disease outbreaks caused by Bacillus cereus, Clostridium perfringens and Staphylococcus aureus - United States, 1998-2008.” *Clinical Infectious Diseases* 57 (2013): 425-433.

Bergdoll, M.S., Crass, B.A., Reiser, R.F., Robbins, R.N., Davis, J.P. “A new staphylococcal enterotoxin, enterotoxin F, associated with toxic-shock-syndrome Staphylococcus aureus isolates.” *Lancet* 1 (1981): 1017-1021.

Bi, X., Tey, L., Leong, C., Quek, R., Henry, C.J. “Prevalence of Vitamin D Deficiency in Singapore: Its Implications to Cardiovascular Risk Factors.” *PLoS One* 22 (2016): e0147616.

Biesalski, H.-K. “Meat as a component of a healthy diet — are there any risks or benefits ifmeat is avoided in the diet?” *Meat Science* 70 (2005): 509-524.

Blais, B.W., Turner, G., Sooknanan, R., Malek, L.T. “A nucelic acid sequence-based amplification system for detection of Listeria monocytogenes hlyA sequences.” *Applied environmental microbiology* 63 (1997): 310-313.

Bohach, G.A., Fast, D.J., Nelson, R.D., Schlievert, P.M. “Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses.” *Critical reviews in microbiology* 17 (1990).

Bolton, D.J. “Campylobacter virulence and survival factors.” *Food Microbiology* 48 (2015): 99 - 108.

Bothwell, T.H., Charlton, R.W. “A general approach to the problems of iron deficiency and iron overload in the population at large.” *Seminars in Hematology* 19 (1982): 54-67.

Brakstad, O.G., Aasbakk, K., Maeland, J.A. “Detection of Staphylococcus aureus by polymerase chain reaction amplification of the nuc gene.” *Journal of Clinical Microbiology* 30 (1992): 1654-1660.

Brenner, D.R., Brockton, N.T., Kitsopoulos, J., Cotterchio, M., Boucher, B.A., Courneya, K.S., Knight, J.A., Olivotto, I.A., Quan, M.L., Friedenreich, C.M. “Breast cancer survival among young women: a review of the role of modifiable lifestyle factors.” *Cancer Causes Control* 12 (2016): Epub ahead of print.

Burt, D.A., Eklud, S.A., Morgan, K.J., Larkin, F.E., Guire, K.E., Brown, L.O., Weintraub, J.A. “The effects of sugars intake and frequency of ingestion on dental caries increment in a three-year longitudinal study.” *J Dent Res* 67 (1988): 1422-1429.

Butaye, P., Devriese, L.A., & Haesebrouck, F. “Differences in antibiotic resistance patterns of Enterococcus faecalis and Enterococcus faecium strains isolated from farm and pet animals.” *Antimicorbial Agents and Chemotherapy* 45 (2001): 1374-1378.

Byberg, L., Bellavia. A, Larsson, S.C., Orsini, N., Wolk, A., Michaëlsson, K. “Mediterranean Diet and Hip Fracture in Swedish Men and Women.” *J Bone Miner Res.* 27 (2016): [Epub ahead of print].

CAC. *Hazard Analysis and Critical Control Point (HACCP) system and guidelines for its application.* Codex Alimentarius Commission Food Hygiene Basic Texts Revision 4., 2003.

Cai, H.Y., Muckle, C.A., Prescott, J.F., Chen, S. “Development of a novel protein microarray method for serotyping Salmonella enterica strais.” *Journal of Clinical Microbiology* 43 (2005): 3427-3430.

Campos, A. K. C., Cardonha, Â. M. S., Pinheiro, L. B. G., Ferreira, N. R., Azevedo, P. R. M. d., Stamford, T. L. M. “Assessment of personal hygiene and practices of food handlers in municipal public schools of Natal, Brazil.” *Food Control* 20 (2009): 807-810.

Carraco, E., Morales-Rueda, A., García-Gimeno, R.M. “Cross-contaminantion and recontamination by Salmonella in foods: A review.” (Food Research International) 45 (2012): 545-556.

Castell, S.G., Rodrigo, P.C., Cruz, N.J., Bartrina, A.J. “Household Food Insecurity Access Scale (HFIAS).” *Nutricion Hospitalaria* 31 (February 2015): 272-278.

CDC. *Annual Report.* 2008.

CDC. “Foodborne Outbreak Investigations.” Centers for Disease Control and Prevention. *http://www.cdc.gov/outbreaknet/investigations/figure_food_production.html.* Atlanta, 2011.

CEC. “Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs.” *Official Journal of the European Union* , 2005.

CEHOG. *Safe Food Handling for bucters.* Crown Copyright, 2012.

CFIA. *Canadian Food Inspection Agency.* 30 09 2014. http://www.inspection.gc.ca/food/non-federally-registered/product-inspection/inspection-manual/eng/1393949957029/1393950086417?chap=5 (accessed 03 21, 2016).

Chaibenjawong, P., Foster, SJ. “Desiccation tolerance in Staphylococcus aureus.” *Archives of Microbiology* 193 (2011): 125-135.

Chen, C.C., Wang, Y.H., Wu, K.Y. “Consumption of bovine spongiform encephalopathy (BSE) contaminated beef and the risk of variant Creutzfeldt-Jakob disease. .” *Risk Anal.* 33 (2013): Epub.

Chen, Y., Zhang, W., Knabel, S.J. “Multi-virulence-locus sequence typing clarifies epidemiology of recent listeriosis outbreaks in the United States.” *Journal of clinical microbiology* 43 (2005): 5291-5294.

Chen, Y., Zhang, W., Knabel, S.T. “Multi-virulence-locus sequence typing identifies single nucleotide polymorphisms wich differentiate epidermic clones and outbreak strains of Listeria monocytogenes.” *Journal of clinical microbiology* 45 (2007): 835-846.

Chesney, R. W., Helms, R. A., Christensen, M., Budreau, A. M., Han, X., Sturman, J. A. “The role of taurine in ingant nutrition.” *Advances in Experimental Medicine and Biology* 442 (1998): 463-476.

Churchill, R.L.T., Lee, H., Hall, J.C. “Detection of Listeria monocytogenes and the toxin listeriolysin O in food.” *Journal of Microbiological Methods* 64 (2006): 141-170.

Cinelli, R.L., O?Dea, J.A. “Obesity Prevention Programs in Children: Impact on Weight, Shape and Food Concern.” *Curr Obes Rep.* 3 (2016): Epub ahead of print.

Clark, M. *Foodborne illness - Common Bacteria and Viruses that Cause Food Poisoning.* Seattle, WA: OutBreak, 2016.

Cocolin, L., Rantsiou, K., Iacumin, L., Cantoni, C., Comi, G. “Direct identification in food samples of Listeria spp. and Listeria monocytogenes by molecular methods.” *Applied environmental microbiology* 68 (2002): 6273-6282.

Conteras, J. *Alimentación y religión.* Parc Científic de Barcelona, Observatório de la Alimentación., Barcelona: Universidad de Barcelona, 2007.

Cossart, P. “Listeriology (1926-2007): the rise of a model pathogen.” *Microbes and infections* 9 (2007): 1143-1146.

Court, L., Hauw, J.J. “[Doctor Francoise Cathala and history of prions diseases].” *Rev Neurol (Paris).* 171 (2015): 805-811.

Crump, J.A., Luby, S.P., Mintz, E.D. “The global burden of typhoid fever.” *Bulletin of the World Health Organisation* 82 (2004): 346-53.

D'Agostino, M., Wagner, M., Vazquez-Boland, J.A., Kuchta, T., Karpiskova, R., Hoorfar, J., Novella, S., Scortti, M., Ellison, J., Murray, A., Fernandes, I., Kuhn, M., Pazlarova, J., Huevelink, A., Cook, N. “A validated PCR-based method to detect Listeria monocytogenes using raw milk as a food model - towards an international standard.” *Journal of food protection* 67 (2004): 1646-1655.

Dalgaard, R., Halberg, N., Hermansen, J.E. *Danish pork production - An environmental assessment.* Faculty of Agricultural Sciences, University of Aarhus, DJF Animal Science, 2007.

D'Aoust, J.Y. *Salmonella species. In: Food Microbiology – Fundamentals and Frontiers.* Washington DC, 1997.

Datta, S., Akter, A., Shah, I.G., Fatema, K., Islam, T.H., Bandyopadhyay, A., Khan, Z.U.M., Biswas, D. “Microbiological Quality Assessment of Raw Meat and Meat Products, and Antibiotic Susceptibility of Isolated Staphylococcus aureus.” *Agriculture Food & Analytical Bacteriology* 2 (2012): 187-194.

De Boeck, E., Jacxsens, L., Bollaerts, M., Uyttendaele, M., Vlerick, P. “Interplay between food safety climate, food safety management system and microbiological hygiene in farm butcheries and affiliated butcher shops.” *Food Control* 65 (2016): 78-91.

de Oliveira Otto, M.C., Afshin, A., Micha, R., Khatibzadeh, S., Fahimi, S., Singh, G., Danaei, G., Sichieri, R., Monteiro, C.A., Louzada, M.L., Ezzati, M., Mozaffarian, D. “The Impact of Dietary and Metabolic Risk Factors on Cardiovascular Diseases and Type 2 Diabetes Mortality in Brazil.” *PLoS One* 11 (2016): e0151503.

De Rycke, J., Milon, A., Oswald, E. “Necrotoxic Escherichia coli (NTEC): two emerging categories of human and animals pathogens.” *Veterinary research* 30 (1999): 221-234.

Delhalle, L., Saegerman, C., Farnir, F., Korsak, N., Maes, D., Messens, W., De Sadeleer, L., De Zutter, L., Daube, G.,. “Salmonella surveillance and control at post-harvest in the Belgian pork meat chain.” *Food Microbiology* 26 (2009): 265-271.

Den Bakker, H., Fortes, E.D., Wiedmann, M. “Multilocus sequence typing of outbreak-associated Listeria monocytogenes isolates to identify epidemic clones.” *Foodborne Pathogens and Disease* 7 (2010): 257-265.

Dhama, K., Rajagunalan, S., Chakraborty, S., Verma, A.K., Kumar, A., Tiwari, R., Kapoor, S. “Food-borne pathogens of animal origin-diagnosis, prevention, control and their zoonotic significance: a review.” *Pakistan Journal of Biological Sciences* 16 (2013): 1076-1085.

Dho-Moulin, M., Fairbrother, J.M. “Avian pathogenic Escherichia coli (APEC).” *Veterinary research* 30 (1999): 299-316.

Djekic, I. “Environmental impact of meat industry – current status and future perspectives.” *Procedia Food Science* 5 (2015): 61-64.

DL. “Diário da República, 1.ª série - .º 206 - 23 de Outubro de 2008.” *Diário da República*, 2008.

—. “Decreto de Lei 1a série No 146 Dec-lei 147/2006 31 Julho 2006.” *Diário da Républica*, 31 Julho 2006.

Doumith, M., Bucherieser, C., Glaser, P., Jacquet, C., Martin, P. “Differentiation of the major Listeria monocytogenes serovars by multiplex PCR.” *Journal of clinical microbiology* 42 (2004): 3819-3822.

EC. “Commission regulation (EC) Nº 1069/2009 health rules as regards animal by-products and derived products not intended for human consumption .” *Official Journal of the European Union*, 2009.

—. “Commission regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs.” *Official Journal of the European Union*, 2005: 1-26.

—. “REG (EC) No835/2004. 2002 Regulation (2004). EUOJ L139/55.” 2004.

Echeita, M.A., Herrera, S., Garaizar, J., Usera, M.A. “Multiplex PCR-based detection and identification of the most common Salmonella second-phase flagellar antigens.” *Research in microbiology* 153 (2002): 107–113.

EFSA. European Food Safety Authority. http://www.efsa.europa.eu/en/topics/topic/parasitesinfood (accessed 03 22, 2016).

EFSA. “Analysis of the baseline survey on the prevalence of Campylobacter in broiler batches and of Campylobacter and Salomnella on broiler carcasses in the EU, 2008.” *EFSA Journal*, 2010: 1503-1602.

EFSA and ECDC. “The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014.” (EFSA Journal) 13 (2015): 4329,191.

EFSA. *Bovine Spongiform Encephalopathy (BSE).* 21 07 2014. https://www.efsa.europa.eu/en/topics/topic/bovinespongiformencephalopathy (accessed 08 12, 2016).

EFSA. “EU summary report on zoonoses, zoonotic agents and food-borne outbreaks 2013.” EFSA European Food Safety Authoruty, 2015, 162.

EFSA. “Opinion of the scientific panel on contaminants in the food chain on a request from the European Commission related to hormone residues in bovine meat and meat products.” *The EFSA Journal* 510 (2007): 1-62.

EFSA. *Report for 2013 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products.* European Food Safety Authority, Parma: EFSA supporting publication, 2015, 69pp.

EFSA. *The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012.* European Centre for Disease Prevention and Control, European Food Safety Authority, EFSA Journal, 2015.

EFSA. *The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012.* EFSA, 2012.

EHOA. *Butchers' Booklet - Guidance on Food Safety and Labelling Requirements relating to Butchers' Counters.* Vol. 1. Environmental Health Officers' Association, 2011.

Eisenbrand, G. “Current issues and perspectives in food safety and risk assessment.” *Hum Exp Toxicol.* 34 (2015): 1286-1290.

Ellis, J.D., Arendt, S.W., Strohbehn, C.H., Meyer, J., Paez, P. “Varying influences of motivation factors on employees' likelihood to perform safe food handling practices because of demographic differences.” *J Food Prot.* 73 (2010): 2065-2071.

Epps, S.V.R., Harvey, R.B., Hume, M.E., Phillips, T.D., Anderson, R.C., Nisbet, D.J. “Foodborne Campylobacter: Infections, Metabolism, Pathogenesis and Reservoirs.” *International Journal of Environmental Research and Public Health* 10 (Nov 2013): 6292-6304.

Escherich, T. “Die Darmbacterien des Neugeborenen und Saglings.” *Fortschritte der Medicin* 3 (1885).

Esposito, K., Maiorino, MI., Bellastella, G., Panagiotakos, D.B., Giugliano, D. “Mediterranean diet for type 2 diabetes: cardiometabolic benefits.” *Endocrine.* 9 (2016): [Epub ahead of print].

Esteban, J.I., Oporto, B., Aduriz, G., Just, R.A., Hurtado, A. “Faecal shedding and strain diversity of Listeria monocytogenes in healthy ruminants and swine in Northern Spain.” *BMC Veterinay Research* 5 (2004).

EUFIC. *Food-Based Dietary Guidelines in Europe.* 10 2009. http://www.eufic.org/article/en/expid/food-based-dietary-guidelines-in-europe/ (accessed 03 17, 2016).

European Parliament Debate. “White Paper on Nutrition, Overweight and Obesity-related health issues (debate).” *Debate.* Edited by GUE/NGL Group. Brussels, 24 09 2008.

Eurostat database. *Eurostat database.* 25 11 2015. http://ec.europa.eu/eurostat/statistics-explained/index.php/Overweight_and_obesity_-_BMI_statistics#General_health_statistics_articles (accessed 03 20, 2016).

FAO. *FAO, Animal Production and Health.* Agriculture and Consumer Protection Department. 05 March 2015. http://www.fao.org/ag/againfo/themes/es/meat/background.htm (accessed March 09, 2016).

FAO. “Fats and fatty acids in human nutrition: report of an expert consultation.” *Food and Agriculture Organization of the United Nations*, 2010.

—. *Food and Agriculture Organization of the United Nations .* 2012. http://faostat.fao.org/ (accessed 07 05, 2016).

—. *Food safety and quality.* 19 11 2014. http://www.fao.org/food/food-safety-quality/events-projects/event/detail/en/c/266111/ (accessed 05 10, 2016).

—. *Meat processing technology for small-to medium-scale producers.* 2016. http://www.fao.org/docrep/010/ai407e/AI407E23.htm (accessed 07 11, 2016).

—. *World Health Day.* 07 04 2015a. http://www.searo.who.int/entity/world_health_day/2015/whd-what-you-should-know/en/ (accessed 07 11, 2016).

Fara, G.M. “Nutrition between sustainability and quality.” *Ann Ig.* 27 (2015): 693-704.

Farber, J.M., Peterkin, P.I. “Listeria monocytogenes, a foodborne pathogen.” *Microbiology and molecular biology reviews* 55 (1991): 476-511.

Fatica, M.K., Keith R. S. “Salmonella and produce Survival in the plant environment and implications in food safety.” *Virulence*, November/December 2011: 573-579.

FDA. *FDA Food Code 2009: Annex 3 - Public Health Reasons / Administrative Guidelines - Chapter 3, Food.* Edited by U.S. Food and Drug Administration. Silver Spring, 2009b.

Fernández, H., Vera, F., Villanueva, M.P., García, A. “Occurence of Campylobacter species in healthy well-nourished and malnourished children.” *Brazilian Journal Microbiology* 39 (2008): 1-310.

Fink, R., Filip, S.M., Oder, M., Jevs̆nik̨ M. *Microbial pathogens and strategies for combating them: science, technology and education.* Formatex, 2013.

Fisher, A., Eiff, C., Kuczius, T., Omoe, K., Peters, T., Becker, K. “A quantitative real-time immuno-PCR approach for detection of staphylococcal enterotoxins.” *Journal of Molecular Medicine*, May 2007: 461-469.

Fitzgerald, C., Collins, M., van Duyne, S., Mikoleit, M., Brown, T., Fields, P. “Multiplex, bead-based suspension array for molecular determination of common Salmonella serogroups.” *Journal of clinical microbiology* 45 (2007): 3323–3334.

Flint, S., Drocourt, J.L., Walker, K., Stevenson, B., Dwyer, M., Clarke, I., McGill, D. “A rapid, two-hour method for the enumeration of total viable bacteria in samples form commercial milk powder and whey protein concentrate powder manufacturing plants.” *Int. Dairy J.* 16 (2006): 379-384.

Flint, S., Walker, K., Waters, B., Crawford, R. “Description and validation of a rapid (1h) flow cytometery test for enumeratic thermiphilic bacteria in milk powders.” *J. Appl. Microbiol.* 102 (2007): 909-915.

Frank, B.H. “Globalization of Food Patterns and Cardiovascular Disease Risk.” *Circulation* 118 (2008): 1913-1914.

Fry, A.M., Braden, C.R., Griffin, P.M., Hughes, J.M. *Principles and practice of infectious diseases.* Edited by J. E. Bennett, & R. Dolin (Eds.) G. L. Mandell. New York: Elsevier, Churchill Livingston., 2005.

FSA. *Food Hygiene ~ A Guide for Businesses.* Vol. 1. Food Standards Agency, 2013.

Garaizar, J., López-Molina, N., Laconcha, I., Baggesen, D.L., Rementeria, A., Vivanco, A., et al. “Suitability of PCR fingerprinting, infrequent-restriction-site PCR, and pulse-field gel electrophoresis, combined with computerized gel analysis, in library typing of Salmonella enterica serovar Enteritidis.” *Applied Environmental Microbiology* 66 (2000): 5273-5281.

Geissmann, T., Chevalier, C., Cros, M-J., Boisset, S., Fechter, P., Noirot, C., Schrenzel, J., François, P., Vandenesch, F., Gaspin, C., Romby, P. “A search for small noncoding RNAs in Staphylococcus aureus reveals a conserved sequence motif for regulation.” *Nucleic Acids Research* 37 (2009): 7239-7257.

Gerber, P.J., Henderson, B., Opio, C., Mottet, A., Steinfeld, H. “Trackling climate change through livestock - A global assessment of emissions and mitigation opportunities.” Food and Agriculture Organization of the United Nations (FAO), Rome, 2013.

Goldberg, A.M. “Farm animal welfare and human health.” *Curr Environ Health Rep.* 25 (2016): [Epub ahead of print].

Gomes-Neves E., Cardoso CS., Araújo AC., Costa, JMC. “Meat handlers training in Portugal: A survey on lnowledge and practice.” *Food Control* 22 (2011): 501-507.

Gomes-Neves, E., Antunes, P., Tavares, A., Themudo, P., Cardoso, M.F., Gärtner, F., Costa, J.M.C., Peixe, L. “Salmonella cross-contamination in swine abattoirs in Portugal: Carcasses, meat and meat handlers.” *International Journal of Food Microbiology.* 157 (2012): 82-87.

Graffouillère, L., Deschasaux, M., Mariotti, F., Neufcourt, L., Shivappa, N., Hébert, J.R., Wirth, M.D., Latino-Martel, P., Hercberg, S., Galan, P., Julia, C., Kesse-

Guyot, E., Touvier, M. “The Dietary Inflammatory Index Is Associated with Prostate Cancer Risk in French Middle-Aged Adults in a Prospective Study.” *J Nutr* 9 (2016): Epub ahead of print.

Grimont, A.D., Well, F.X. *Antigenic formulae of the Salmonella serovars.* 9th. WHO Collaborating Centre for Reference and Research on Salmonella, 2007.

Gunasekera, T.S., Veal, D.A., Attfield, P.V. “Potential for broad applications of flow cytometry and fluorescence techniques in microbiological and somatic cell analyses of milk.” *Int. J. Food Microbiol.* 85 (2003): 269-279.

Haris, M. *Bueno para comer.* Madrid: Alianza editorial, 1989.

Hasegawa, M., Iwabuchi, E., Yamamoto, S., Muramatsu, M., Takashima, I., Hirai, K. “Prevalence and characteristics of Listeria monocytogenes in feces of black beef cattle reared in three geographically distant areas in Japan.” *Foodborne pathogene and disease* 11 (2014): 96-103.

Hasse, J.K., Didelot, X., Lecuit, M., Korkeala, H., L.monocytogenes MLST Study Group., Achtman, M. “L. monocytogenes MLST Study Group, Achtman M. The ubiquitous nature of Listeria monocytogenes clones: a large scale multilocus sequence typing study.” *Environmental microbiology* 16 (2014): 405-416.

Hendriksen, R.S., Vieira, A.R., Karlsmose, S., Lo Fo Wong, D.M., Jensen, A.B., Wegener, H.C., Aarestrup, F.M. “Global monitoring of Salmonella serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: Results of quality assured laboratories from 2001 to 2007.” *Foodborne Pathogens and Disease* 8 (2011): 887-900.

Hennekinne, J.A., Buyser, M.L., Dragacci, S. “Staphylococcus aureus and its fodd poisoning toxins.” *FEMS Microbiology Reviews* 36 (2012): 815-836.

Hernandez-Milian, A., Payeras-Cifre, A. “What Is New in Listeriosis?” *Biomed Research International* 14 (2014): 1-14.

Herrera-León, S., McQuiston, J.R., Usera, M.A., Fields, P.I., Garaizar, J., Echeita, M.A. “Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of Salmonella spp.” *Journal of clinical microbiology* 42 (2004): 2581-2586.

Herrera-León, S., Ramiro, R., Arroyo, M., Díez, R., Usera, M.A., Echeita, M.A. “Blind comparison of traditional serotyping with three multiplex PCRs for the identification of Salmonella serotypes.” *Research in microbiology* 158 (2007): 122-127.

Hierro, N., Esteve-Zarzoso, B., Gonzalez, A., Mas, A., Guillamon, J.M. “Real-time quantitative PCR (QPCR) and reverse transcription-QPCR for detection and enumeration of total yeasts in wine.” *Appl. Environ. Microbiol.* 72 (2006): 7148-7155.

Hierro, N., Esteve-Zarzoso, B., Mas, A., Guillamon, J.M. “Monitoring of Saccharomyces and Hanseniaspora populations during alcoholic fermentation by real-time quantitative.” *FEMS Yeast Res.* 7 (2007): 1340-1349.

Higgs, J.D. “The changing nature of red meat: 20 years of improving nutritional quality.” *Trends in Food Science and Technology.* 11 (2000): 85-95.

Hooper, L., Abdelhamid, A., Moore, H.J., Douthwaite, W., Skeaff, C.M., Summerbell, C.D. “Effect of reducing total fat intake on body weight: systematic review and meta-analysis of randomised controlled trials and cohort studies.” *BMJ*, 2012: 345-e7666.

Hoorfar, J. *Global safety of fresh produce: A handbook of best-practice examples, innovative commercial solutions and case studies.* Edited by Technology and Nutrition Woodhead Publishing Series in Food Science. Woodhead Publishing, 2014.

Hori, K., Matsumoto, S. “Bacterial adhesion: From mechanisms to control.” *Biochemical Engineering Journal.* 48 (2010): 424-434.

Hough, A.J., Harbison, S.-A., Savill, M.G., Melton, L.D., Fletcher, G. “Rapid enumeration of Listeria monocytogenes in artificially contaminated cabbage using

real time polymerase chain reaction." *Journal of food protection* 65 (2002): 1329-1332.

Hudson, J.A., Lake, R.J., Savill, M.G., Scholes, P., McCormick, R.E. "Rapid detection of Listeria monocytogenes in ham samples using immunomagnetic separation followed by polymerase chain reaction." *Journal of applied microbiology* 90 (2001): 614-621.

ICMSF. *Microorganisms in Food 7.* International Commission on Microbiological Specifications for Foods. New York: Kluwer Academic/Plenum Publishers, 2002.

IFC. *Meat processing environmental, health and safety guidelines.* Edited by World Bank Group. Washington DC: W.B.G.-I.F. Corporation (Ed.), 2007.

IFD. *IFD Diabetes Atlas, Seventh edition.* Brussels: Internation Diabetes Federation (IFD), 2015.

IFT. "Bacteria associated with foodborne diseases." Institute of food technologist (IFT), Chicago, 2004.

INE. *Instituto Nacional de Estatística.* 05 2015. https://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_indicadores&indOcorrCod=0000211&contexto=bd&selTab=tab2 (accessed 04 07, 2016).

Ingianni, A., Floris, M., Palomba, P., Madeddu, M.A., Quartuccio, M., Pompei, R. "Rapid detection of Listeria monocytogenes in foods, by a combination of PCR and DNA probe." *Molecular and cellular probes* 15 (2001): 275-280.

IPPC. "Reference Document on Best Available Techniques in the Food, Drink and Milk Industries." Integrated Pollution Prevention and Control., European Comission, Seville, 2006.

Ismaïl, R., Aviat, F., Michel, V., Le Bayon, I., Gay-Perret, P., Kutnik, M., Fédérighi, M. "Methods for Recovering Microorganisms from Solid Surfaces Used in the Food Industry: A Review of the Literature." *Int J Environ Res Public Health* 10 (2013): 6169-6183.

ISO. “Food Safety management systems—requirements for any organization in the food chain.” BS EN ISO 22000. 2005, London, 2005.

—. “ISO 22174:2005 Microbiology of food and animal feeding stuffs -- Polymerase chain reaction (PCR) for the detection of food-borne pathogens -- General requirements and definitions.” International Organization for Standardization, 2014.

—. “ISO, 1996. ISO 11290-1:1996. Microbiology of Food and Animal Feeding Stuffs - Horizontal Method for the Detection and Enumeration of Listeria monocytogenes - Part 1: Detection Method.” Geneva.

—. “Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) -- Part 1: Technique using Baird-Parker agar medium.” *ISO 6888-1:1999.* 1999a.

—. “Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) -- Part 2: Technique using rabbit plasma fibrinogen agar medium.” *ISO 6888-2:1999.* 1999b.

Jasson, V., Jacxsens, L., Luning, P., Rajkovic, A., Uyttendaele, M. “Alternative microbial methods: An overview and selection criteria.” *Food Microbiology* 27 (2010): 710-730.

Jay, J.M. *Gastroenterites estafilocócica. Microbiología moderna de los alimentos.* 4. Zaragoza: Editorial Acribia S.A., 2001.

Jensen, C.O. “Ueber die Kalberruhr und deren Aetiologie.” *Mh. Tierheilkd* 4 (1893): 97-124.

Jensen, D.A., Danyluk, M.D., Harris, L.J., Schaffner, D.W. “Quantifying the effect of hand wash duration, soap use, ground beef debris, and drying methods on the removal of Enterobacter aerogenes on hands.” *J Food Prot.* 78 (2015): 685-690.

Jianu, C., Chis, C. “Study on the hygiene knowledge of food handlers working in small and medium-sized companies in western Romania.” *Food Control* 26 (2012): 151-156.

Johnson, W.M., Tyler, S.D., Ewan, E.P., Ashton, F.E., Pollard, D.R., Rpzee, K.R. “Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in Staphylococcus aureus by the polymerase chain reaction.” *Journal of Clinical Microbiology* 29 (1991): 426-430.

Josefsen, M.H., Bhunia, A.K., Engvall, E.O., Fachmann, M.S., Hoorfar, J. “Monitoring Campylobacter in the poultry production chain - From culture to genes and beyond.” *Journal of microbiological methods* 112 (2015): 118-125.

Jumaa, P.A. “Hand hygene: simple and complex.” *International Journal of Infectious Diseases* 9 (2005): 3-14.

Jun, S., Ha, K., Chung, S., Joung, H. “Meat and milk intake in the rice-based Korean diet: impact on cancer and metabolic syndrome.” *Proc Nutr Soc* 15 (2016): 1-11.

Jung, Y.S., Frank, J.F., Brackett, R.E., Chen, J. “Polymerase chain reaction detection of Listeria monocytogenes in frankfurters using oligonucleotide primers targetting the genes encoding internalin AB.” *Journal of food protection* 66 (2003): 237-241.

Kadariya, J., Smith, T.C., Thapaliya, D. “Staphylococcus aureus and Staphylococcal Food-Borne Disease: An Ongoing Challenge in Public Health.” *BioMed Research International*, April 2013: 1-9.

Kaper, J.B., Nataro, J.P., Mobley, H.L. “Pathogenic Escherichia coli.” *Nature Reviews Microbiology* 2 (2004): 123-140.

Kappeler, R., Eichholzer, M., Rohrmann, S. “Meat consumption and diet quality and mortality in NHANES III.” *European Journal of Clinical Nutrition* 67 (2013): 698-606.

Kauffmann, F. “The serology of the coli group.” *Journal of immunology* 57 (1947): 71-100.

Kerouanton, A., Marault, M., Lailler, R., Weill, F.X., Feurer, C., Espié, E., Brisabois, A. “Pulsed-field gel electrophoresis subtyping database for foodborne Salmonella enterica serotype discrimination.” *Foodborne pathogenese and disease* 4 (2007): 293-303.

Ketley, J.M. “Pathogenesis of enteric infection by Campylobacter.” *Microbiology* 143 (1997): 5-21.

Keys, A. “Mediterranean diet and public health: personal reflections.” *Am J Clin Nutr* 61 (1995): 1321S-1323S.

Kibret, M., Abera, B. “The Sanitary Conditions of Food Service Establishments and Food Safety Knowledge and Practices of Food Handlers in Bahir Dar Town.” *Ethiop J Health Sci.* 22 (2012): 27-35.

Kim, S., Frye, J.G., Hu, J., Fedorka-Cray, P.J., Gautom,R., Boyle, D.S. “Multiplex PCR-Based Method for Identification of Common Clinical Serotypes of Salmonella enterica subsp. enterica.” *Journal of Clinical Microbiology* 44 (2006): 3608–3615.

King, S., Adams, M.C. “ncidence in Campylobacter in prcessed poultry: Is it a concern for human health.” *Journal of Food Safety* 28 (2008): 376-388.

Korsgaard, H., Madsen, M., Feld, N.C., Mygind, J., Hald, T. “The effects, costs and benefits of Salmonella control in the Danish table-egg sector.” *Epidemiology Infection* 137 (2009): 828-836.

Kouris-Blazos, A., Belski, R. “Health benefits of legumes and pulses with a focus on Australian sweet lupins. .” *Asia Pac J Clin Nut* 25 (2016): 1-17.

Kouvari, M., Tyrovolas, S., Panagiotakos, D.B. “Red meat consumption and healthy ageing: A review.” *Maturitas*, 2015: 1-8.

Kuroda, M., Sakaue, H. “[Role of vitamin D and calcium in obesity and type 2 diabetes].” *Clin Calcium.* 26 (2016): 349-354.

Kusumaningrum, H.D., van Putten, M., Rombouts, F.M., Beumer, R.R. “Effects of antibacterial dishwashing liquid on foodborne pathogens and competitive microorganisms in kitchen sponges.” *Journal of Food Protection* 65 (2002): 61-65.

Lübeck, P.S., Hoorfar, J. “PCR technology and applications to zoonotic food-borne bacterial pathogens.” *Methods in molecular biology* 216 (2003): 65-84.

Lammerding, A.M. “An overview of microbial food safety risk assessment.” *Journal of Food Protection* 60 (1997): 1420-1425.

Larsen, C.S. “Animal source foods to improve micronutrient nutrition and human function in developing countries animal source foods and human health during evolution.” *Health (San Francisco)* 1 (2003): 3893-3897.

Leclercqemail, A., Charlier, C., Lecuit, M. “Global burden of listeriosis: the tip of the iceberg.” *The Lancet Infectious Diseases* 14 (2014): 1027–1028.

Lee, J., Kim, S.Y., Hwang, K.J., Ju, Y.R., Woo, H-J. “Prion Diseases and Trnsmissible Zoonotic Diseases.” *Osong Public Health Res Perspect.* 4 (2013): 57-66.

Lehner, A., Loncarevic, S., Wagner, M., Kreike, J., Brandl, E. “A rapid differentiation of Listeria monocytogenes by use of PCR-SSCP in the listeriolysin O (hlyA) locus.” *Journal of microbiological methods* 34 (1999): 165-171.

Leroy, F., Praet, I. “Meat traditions. The co-evolution of humans and meat.” *Appetite* 90 (2015): 200-211.

Li, F., Duan, F., Zhao, X., Song, C., Cui, S., Dai, L. “Red Meat and Processed Meat Consumption and Nasopharyngeal Carcinoma Risk: A Dose-response Meta-analysis of Observational Studies.” *Nutr Cancer.* 1 (2016): [Epub ahead of print].

Lindemann, I.L., Oliveira, R.R., Mendoza-Sassi, R.A. “[Difficulties in ensuring a healthy diet among primary health care users and associated factors].” *Cien Saude Colet.* 21 (2016): 599-610.

Liu, D., Ainsworth, F., Austin, F., Lawrence, M. “Characterization of virulent and avirulent Listeria monocytogenes strains by PCR amplification of putative transcriptional refulator and internalin genes.” *Journal of medical microbiology* 52 (2003): 1065-1070.

Loir, Y., Baron, F., Gautier, M. “Staphylococcus aureus and food poisoning.” *Genetics and Molecular Research* 2 (2003): 63-76.

Lomonaco, S., Knabel, S.J., Dalmasso, A., Civera, T., Bottero, M.T. “Novel multiplex single nucleotide polymorphism-based method for identifying epidemic clones of Listeria monocytogenes.” *Applied environmental microbiology* 77 (2011): 6290-6294.

Lopman B.A., Reacher M.H., van Duijnhoven Y., Hanon F.X., Brown D., Koopmans M. “Viral gastroenteritis outbreaks in Europe, 1995–2000.” *Emerging Infectious Dieseases* 9 (2003): 90-96.

Lowy, F.D. “Staphylococcus aureus Infections.” *The New England Journal of Medicine* 78 (August 1998): 520-532.

Lunge, V.R., Miller, B.J., Livak, K.J., Batt, C.A. “Factors affecting the performance of 5' nuclease PCR assays for Listeria monocytogenes detection.” *Journal of microbiological methods* 51 (2002): 361-368.

Luning, P.A., Kirezieva, K., Hagelaar, G., Rovira, J., Uyttendaele, M., Jacxsens, L. “Performance assessment of food safety management systems in animalbased food companies in view of their context characteristics: a European study.” *Food Control* 49 (2015): 11-22.

López, V., Ortiz, S., Corujo, A., López, P., Posa, D., Navas, K., Moreno, R., Martinez-Suares, J. “Different contamination patterns of lineage I and II strains of Listeria monocytogenes in a Spanish broiler abattoir.” *Poultry science* 87 (2008): 1874-1882.

López, V., Suaréz, M., Chico-Calero, I., Navas, J., Martinez-Suarez, J. “Listeria monocytogenes en alimentos: son todos los aislamientos igual de virulentos?” *Revista argentina de microbiología* 8 (2006): 224-234.

Mäntynen, V., Niemelä, S., Kaijalainen, S., Pirhonen, T., Lindström, K. “MPN-PCR-quantification method for staphylococcal enterotoxin c1 gene from fresh cheese.” *International journal of food microbiology* 36 (1997): 135-143.

Maćkowiak, K., Torlińska-Walkowiak, N., Torlińska, B. “Dietary fibre as an important constituent of the diet.” *Postepy Hig Med Dosw (Online)* 25 (2016): 104-109.

Mainil, J. *Facteurs de virulence et propriétés spécifiques des souches invasives d'Escherichia coli: I) Les adhésines et facteurs de colonisation.* Vol. 147. 2003c.

Majowicz, S.E., Musto, J., Scallan, E., Angulo, F.J., Kirk, M., O'Brien, SJ., Jones, T.F., Fazil, A., Hoekstra, R.M. “The global burden of nontyphoidal Salmonella gastroenteritis.” *Clinical infectious diseases* 50 (Mar 2010): 882-889.

Malorny, B., Bunge, C., Guerra, B., Prietz, S., Helmuth, R. “Molecular characterisation of Salmonella strains by an oligonucleotide multiprobe microarray.” *Molecular and cellular probes* 21 (2007): 56-65.

Mann, N. “Meat in the human diet: An anthropological perspective.” *Nutrition and Dietetics* 64 (2007): S102-S107.

Matsuoka, A., Sorenson, J. “Human consequences of animal exploitation: needs for redefining social welfare.” *Jornal os Sociology & Social Welfare* XL (2013): 7-32.

Maughan, C., Chambers Iv, E., Godwin, S., Chambers, D., Cates, S., Koppel, K. “Food Handling Behaviors Observed in Consumers When Cooking Poultry and Eggs.” *J Food Prot.* 79 (2016): 970-977.

Maurer, J. *PCR Methods in Food.* New York: Springer Science Business Media Inc., 2006.

McCartan, C., Perryman, S. “Evaluation of a nutrition education and training (NET) workshop for school personnel.” *School Food Service Research Review* 6 (1982): 109.

McCormick, J.K., Yarwood, J.M., Schlievert, P.M. “Toxic shock syndrome and bacterial superantigens: an update.” *Annual Review Microbiology* 55 (2001): 77-104.

McIntyre, L., Vallaster, L., Wilcott, L., Henderson, S.B., Kosatsky, T. “Evaluation of food safety knowledge, attitudes and self-reported hand washing practices in FOODSAFE trained and untrained food handlers in British Columbia, Canada.” *Food Control* 30 (2013): 150-156.

McLauchlin, J., Mitchell, R., Smerdon, W., Jewell, K. “Listeria monocytogenes and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods.” *International journal of food microbiology* 92 (2004): 15-33.

McQuiston, J.R., Fields, P.I., Tauxe, R.V., Logsdon, Jr., J.M. “Do Salmonella carry spare tyres?” *Trends Microbiology* 12 (2008): 142-148.

Mead P.S., Slutsker L., Dietz V., McCaig L.F., Bresee J.S., Shapiro C., Griffin P.M., Tauxe R.V. “Food-related illness and death in the United States.” *Emerging Infectious Dieseases* 5 (1999): 607-625.

Mertes, F., Biens, K., Lehrach, H., Wagner, M., Dahl, A. “High-throughput Universal Probe Salmonella Serotyping (UPSS) by nanoPCR.” *Journal of microbiological methods* 83 (2010): 217-223.

Messens, W., Vivas-Alegre, L., Bashir, S., Amore, G., Romero-Barrios, P., Hugas, M. “Estimating the Public Health Impact of Setting Targets at the European Level for the Reduction of Zoonotic Salmonella in Certain Poultry Populations.” *International Journal of Environmental Research and Public Health* 10 (2013): 4836-4850.

Minor, L.L., Popoff , M.Y. “Designation of Salmonella enterica sp. nov., norn. rev., as the Type and Only Species of the Genus Salmonella.” *International Journal of Systematic Bacteriology* 37 (1987): 465-468.

Miya, S., Kimura, B., Sato, M., Takahashi, H., Ishikawa, T., Suda, T., Takakura, C., Fujii, T., Wiedmann, M. “Development of a multilocus variable-number of tandem repeat typing method for Listeria monocytogenes serotype 4b strains.” *International Journal of Food Microbiology* 124 (2008): 239-249.

Mohr, T.B., Juneja, V.K., Thippareddi, H.H., Schaffner, D.W., Bronstein, P.A., Silverman, M., Cook, L.V. Jr. “Assessing the Performance of Clostridium perfringens Cooling Models for Cooked, Uncured Meat and Poultry Products.” *J Food Prot.* 78 (2015): 1512-1526.

Much, P., Pichler, J., Allerberger, F. “Food borne infectious outbreaks, Austria 2005.” *Wien Klin Wochenschr* 119 (2007): 150-157.

Muñoz, A.B., Chaves, J.A., Rodríguez, E.C., Realpe, M.E. “[Listeria monocytogenes in food handlers: a new approach to address the dangers in food industry].” *Biomedica.* 33 (2013): 283-291.

Murase, T.M., Nagato, K., Shirota, H., Katoh, Otsuki, K. “Pulsed-field gel electroforesis-based subtyping of DNA degradation-sensitive Salmonella enterica subsp. enterica serovar Livingstone and serovar Cerro isolates obtained from a chicken layer farm.” *Veterinary Microbiology* 99 (2004): 139-143.

Muscaritoli, M., Amabile, M.I., Molfino, A. “Foods and their components promoting gastrointestinal cancer.” *Curr Opin Clin Nutr Metab Care.* 6 (2016): [Epub ahead of print] .

Nagaraj, S., Ramlal, S., Sripathy, MH., Batra, HV. “Development and evaluation of a novel combinatorial selective enrichment and multiplex PCR technique for molecular detection of major virulence-associated genes of enterotoxigenic Staphylococcus aureus in food samples.” *Journal of Applied Microbiology* 116 (2013): 435-446.

Nagy, B., Fekete, P.Z. “Enterotoxigenic Escherichia coli (ETEC) in farm animals.” *Veterinary research* 30 (1999): 259-284.

Nataro, J.P., Kaper, J.B. “Diarrheagenic Escherichia coli.” *Clinical microbiology reviews.* 11 (1998): 142-201.

NCEZID. “National Salmonella Surveillance Overview.” US Department of Health and Human Services, National Center for Emerging and Zoonotic Infectious Diseases, Atlanta, Georgia, 2011.

NHS. *Food Standards Scotland and the Food Standards Agency in Northern Ireland.* Public Health England in association with the Welsh government. 2016. http://www.nhs.uk/Livewell/Goodfood/Documents/The-Eatwell-Guide-2016.pdf (accessed 06 08, 2016).

NRC. *Diet and Health: Implications for Reducing Chronic Disease Risk. Report of the Committee on Diet and Health, Food and Nutrition Board, Commission on Life Sciences.* Recommendations to Action, Food and Nutrition Board, National Research Council: Institute of Medicine, Washington, D.C.: National Academy Press, 1991, 749pp.

Nyfeldt, A. “Etiologie de la mononucleose infectieuse.” *Comptes Rendus des Seances de la Societe de Biologie.* 101 (1929): 590–591.

O., Salazar. *Bacteria and Yeast Cell Disruption Using Lytic Enzymes.* Vol. 424, in *2D PAGE: Sample Preparation and Fractionation*, 23-34. 2008.

OCDE. *Meat consumption (indicator).* 2015. https://data.oecd.org/agroutput/meat-consumption.htm (accessed 04 21, 2016).

OIE. *Animal Health in the World.* 2016. http://www.oie.int/animal-health-in-the-world/bse-specific-data/number-of-reported-cases-worldwide-excluding-the-united-kingdom/ (accessed 08 12, 2016).

Okwumabua, O., O'Connor, M., Shull, E., Strelow, K., Hamacher, M., Kurzynski, T., Warshauer D. “Characterization of Listeria monocytogenes isolates from food

animal clinical cases: PFGE pattern similarity to strains from human listeriosis cases." *FEMS microbiology letters* 249 (2005): 275-281.

Olsen, J.E., Skov, M.N., Threlfall, E.J., Brown, D.J. "Clonal lines of Salmonella enterica serotype Enteritidis documented by IS200-, ribo-pulsed-field gel electrophoresis and RFLP typing." *Journal of medical microbiology* 40 (1994): 15-22.

OMS. *Food Safety.* 12 2015. http://www.who.int/mediacentre/factsheets/fs399/en/ (accessed 03 21, 2016).

OMS/FAO. *Dieta, Nutrición y Prevención de enfermedades crónicas.* Consulta Mixta OMS/FAO de Expertos en Régimen Alimentario, Nutrición y Prevención de Enfermedades Crónicas, OMS/FAO, World Health Organisation, 2003.

Oostindjer, M., Alexander, J., Amdam, G.V., Andersen, G., Bryane, N.S., Chen, D., Corpet, D.E., De Smet, S., Dragsted, L.A., AnnaHaug, Karlsson, A.H., Kleter, G., de Kokm, T., Kulseng, B., Milkowski, A.L., Martino, R.J., Pajari, A., Paulsen, J.E., Pickova, J., Rudi, K., Sødring, M., Weed, D.L., Egelandsdal, B. "The role of red and processed meat in colorectal cancer development: a perspective." *Meat Science* 97 (2014): 583-596.

Orndorff, P., Hamrick, T., Smoak, I., Havell, E. "Host and bacterial factors in listeriosis pathogenesis." *Veterinary microbiology* 114 (2006): 1-15.

Oses, S.M., Luning, P.A., Jacxsens, L., Santillana, S., Jaime, I., Rovira, J. "Food safety management system performance in the lamb chain." *Food Control* 25 (2012): 493-500.

Painter, J.A., Hoekstra, R.M., Ayers, T., Tauxe, R.V., Braden, C.R., Angulo, F.J., Griffin, P.M. "Attribution of Foodborne Illnesses, Hospitalizations, and Deaths to Food Commodities by using Outbeak Data, United States, 1998-2008." *Emerging Infectious Diseases* 19 (2013): 407-415.

Park, S., Kwak, T., Chang, H. "Evaluation of the food safety training for food handlers in restaurant operations." *Nutrs Res Pract.* 4 (2010): 58-68.

Parnet, P., Paillé, V., Bolaños Jimenex, F., Kaeffer, B., Schaal, B., Bouret, S.G., Amarger, V. “The sensory and nutritional experience of parents guide offsprings' feeding behavior.” *Med Sci (Paris)* 32 (2016): 85-92.

Pereira, P.M.C.C., Vicente, A.F.R.B. “Meat nutritional composition and nutritive role in the human diet.” *Meat Science* 93 (2013): 586-592.

Popoff, M.Y., Le Minor, L. *Formules antigéniques des sérovars de Salmonella.* 8th. Edited by WHO Collaborating Centre for Reference and Research on Salmonella. Paris, 1997.

Postollec, F., Falentin, H., Pavan, S., Combrisson, J., Sohier, D. “Recent advances in quantitative PCR (qPCR) applications in food microbiology.” *Food Microbiology* 28, no. 5 (2011): 1-14.

Prusiner, S.B. *Prion biology and diseases.* Edited by Cold Spring Harbor. 2nd Ed. vols. NY: Cold Spring Harbor Laboratory Press., 2004.

Public Health England. *Detection and enumeration of Campylobacter species.* London: PHE Microbiology Services Food, Wather & Environmental Microbiology Methods Working Group, 2014.

Que, Y.A., Moreillon, P. “Infective endocarditis.” *Nature Review Cardiology* 8 (2011): 322-336.

Raengpradub, S., Wiedmann, M., Boor, K.J. “Comparative analysis os the sigma B-dependent stress responses in Listeria monocytogenes and Listeria innocua strains exposed to selected stress conditions.” *Applied environmental microbiology* 74 (2008): 158-171.

Ramalho, V., Pinto de Moura, A., Cunha, L.M. “Why do small business butcher shops fail to fully implement HACCP?” *Food Control* 49 (2015): 85-91.

Raspor, P., Jevšnik, M. “Good nutritional practice from producer to consumer.” *Critical Reviews in Food Science and Nutrition.* 48 (2008): 276-292.

Rebuffo, A., Schmitt, J., Wenning, M., Stetten, F., Scherer, S. “Reliable and rapid identification of Listeraia monocytogenes and Listeria species by artificial neural network - based fourier transform infrared spectroscopy.” *Applied and environmental microbiology* 72 (2005): 994-1000.

Reckmann, K., Traulsen, I., Krieter, J. “Environmental Impact Assessment – methodology with special emphasis on European pork production.” *J Environ Manage* 107 (2012): 102-109.

Regenstein, J.M., Chaudry, M.M., Regenstein, C.E. “The Kosher and Halal Food Laws.” *Comprehensive Reviews in Food Science and Food Safety* 2 (2003): 111-127.

Reig, M., Toldrá, F. “Veterinary drug residues in meat: Concerns and rapid methods for detection.” *Meat Science* 78 (2007): 60-67.

Remond, E.C., Griffith, C.J. *MEDICAL SCIENCES - Home Food Safety and Consumer Responsability.* Vol. II. II vols. Encyclopedia of Life Support Systems (EOLSS), 2010.

Renehan, A.G., Soerjomataram, I., Tyson, M., Egger, M., Zwahlen, M., Coebergh, J.W., Buchan, I. “Incident cancer burden attibutable to excess body mass index in 30 European countries.” *Int J Cancer* 126 (2010): 692-702.

Rivas, K.D., Canter, D.D. “Effectiveness of talent for training school foodservice employee.” *School Foodservice Research Review* 6 (1982): 113.

Rodrigues, D., Muc, M., Rodrigues, P.R., Pinto, A.M., Padez, C. “Dietary Patterns and Their Socioeconomic and Behavioral Determinants in 6- to 8-Year-Old Portuguese Children.” *Ecol Food Nutr.* 11 (2016): [Epub ahead of print].

Rodríguez-Lázaro, D., Hernández, M., Scortti, M., Esteve, T., Vazquez-Boland, J.-A., Pla, M. “Quantitative detection of Listeria monocytogenes and Listeria innocua by real-time PCR: assessment of hly, iap, and lin02483 targets and AmpliFluor technology.” *Applied environmental microbiology* 70 (2004): 1366-1373.

Rohde, A., Hammerl, J.A., Appel, B., Dieckmann, R., Al Dahouk, S. “FISHing for bacteria in food e A promising tool for the reliable detection of pathogenic bacteria?” *Food Microbiology* 46 (2015): 395-407.

Rohrmann, S., Overvad, K., Bueno-de-Mesquita, H. B., Jakobsen, M. U., Egeberg, R., Tjønneland, A., et al. “Meat consumption and mortality — Results from the European prospective investigation into cancer and nutrition.” *BMC Medicine* 11 (2013): 63.

Rokosz, N., Rastawichi, W., Wolkowicz, T. “Microbiological diagnosis of infections caused by Campylobacter jejuni and Campylobacter coli in humans.” *Postepy higieny i medycyny doswiadcaalnej (Online).* 68 (2014): 48-56.

Rotilio, G., Marchese, E. “Nutritional factors in human dispersals.” *Ann Hum Biol.* 37 (2010): 312-324.

Rugg-Gunn, A.J. *Nutrition and dental health.* Oxford: Oxford Medical Publications, 1993.

Rugg-Gunn, A.J., Hackett, A.F., Appleton, D.R., Jenkins, G.N., Eastoe, J.E. “Relationship between dietary habits and caries increment assessed over two years in 405 English adolescent school children.” *Arch Oral Biol* 29 (1984): 983-992.

Saruta, K., Matsunaga, T., Kono, M., Hoshina, S., Ikawa, S., Sakai, O., Machida, K. “Rapid identification and typing of Staphylococcus aureus by nested PCR amplified ribosomal DNA spacer region.” *FEMS Microbiology Letters* 146 (1997): 271-278.

Sauders, B.D., Wiedmann, M. “Ecology of Listeria species and L. monocytogenes in the natural environment.” In *Listeria, Listeriosis and Food Safety*, by E.T., Marth, E.H. Ryser, 21-54. CRC Press, 2007.

Saunders, S.E., Bartelt-Hunt, S.L., Bartz, J.C. “Prions in the environment: occurrence, fate and mitigation.” *Prion.* 2 (2008): 162-169.

Scallan, E., Hoekstra, R., Angulo, F., Tauxe, R., Widdowson, M., Roy, S.L., Jones, J.L., Griffin, P.M. “Foodborne illness acquired in the United States—major pathogens.” *Emerging Infectious Diseases* 17 (2011): 7-15.

Schmid, M., Walchor, M., Bubert, A., Wagner, M., Wagner, M., Schleifer, K.-H. “Nucleic acid-based, cultivation independent detection of Listeria spp. and genotypes of Listeria monocytogenes.” *FEMS Immunology and medical microbiology* 35 (2003): 215-225.

Schrader, C., Schielke, A., Ellerbroek, L., Johne, R. “PCR Inhibitors - occurrence, properties and removal.” *Journal of applied microbiology* 113 (2012): 1014-1026.

Scott, E., Bloomfield, SF. “The survival and transfer of microbial contamination via cloths, hands and utensils.” *Journal of Applied Microbiology* 68 (1990): 271-278.

Sesto, N., Touchon, M., Andrade, J.M., Kondo, J., Rocha, E.P.C., Arraiano, C.M., Archambaud, C., Westhof, É., Romby, P., Cossart, P. “A PNPase dependent CRISPR System in Listeria.” *PloS Genetic* 10 (2014): e1004065.

Severino, P., Dussurget, O., Vêncio, R.Z., Dumas, E., Garrido, P., Padilla, G., Piveteau, P., Lemaître, J-P., Kunst, F., Glaser, P., Buchrieser, C. “Comparative transcriptome analysis of Listeria monocytogenes strains of the two major lineages reveals differences in virulence, cell wall, and stress response.” *Applied environmental microbiology* 73 (2007): 6078-6088.

Shojaei, H., Shooshtaripoor, J., Amiri, M. “Efficacy of simple hand-washing in reduction of microbial hand contamination of Iranian food handlers.” *Food Research International.* 39 (2006): 525-529.

Silva J., Leite D., Fernandez M., Mena C., Gibbs P.A., Teixeira P. “Campylobacter spp. as a foodborne pathogen: A review. .” *Front. Microbiology* 2 (2011): 1-12.

Sirinavin, S., Jayanetra, P., Thakkinstian, A. “Clinical and prognostic categorization of extraintestinal nontyphoidal Salmonella infections in infants and children.” *Clinical Infectious Disease* 29 (1999): 1151-1156.

Skirrow, M.B. “John McFadyean and the Centenary of the First Isolation of Campylobacter Species.” *Clinical Infectious Diseases* 43 (Nov 2006): 1213-1217.

Smil, V. “Eating meat. Evolution, patterns, and consequences.” *Population and Development Review* 28 (2002): 599-639.

Smith, J.L. “A review of hepatitis E virus.” *Journal of Food Protection* 64 (2001): 572-586.

Snelling, W.J., Matsuda, M., Moore, J.E., Dooley, J.S.G. “Under the microscope Campylobacter jejuni.” *Applied Microbiology* 41 (2005): 1-12.

Soriano, J.M., Prieto, I., Moltó, J.C., Mañes, J. “A review of the application of the hazard analysis and critical control point system to salads served in the restaurant of Valencia University.” *International Journal of Food Science and Technology* 40 (2005): 333-336.

Stach, Cs., Herrera, A., Schlievert, PM. “Staphylococcal superantigens interact with multiple host receptors to cause serious disease.” *Immunologic Research* 59 (2014): 177-181.

Stecksen-Blicks, C., Gustafsson, L. “Impact of oral Hygiene and use of fluorides on caries increment in children during one year.” *Community Dentistry and Oral Epidemiology* 14 (1986): 185-189.

Straub, J.A., Hertel, C., Hammes, W.P. “A 23S rDNA-targeted polymerase chain reaction-based system for detection of Staphylococcus aureus in meat starter cultures and dairy products.” *Journal of food protection* 62 (1999): 1150-1156.

Suckling, R.J., Swift, P.A. “The health impacts of dietary sodium and a low-salt diet.” *Clin Med (Lond)* 15 (2015): 585-588.

Sue, D., Fink, D., Wiedmann, M., Boor, K.J. “Sigma B-dependent gene induction and expression in Listeria monocytogenes during osmotic and acid stress conditions simulating the intestinal environment.” *Microbiology* 150 (2004): 3843-4455.

Suzuki, H., Yamamoto, S. “Campylobacter contamination in retail poultry meats and by-products in the world: a literature survey.” *J Vet Med Sci.* 71 (2009): 255-261.

Swaminathan, B., Gerner-Smidt, P. “Forum: The epidemiology of human listeriosis.” *Microbes and Infection* 9 (2007): 1236-1243.

Syne, S.M., Ramsubhag, A., Adesiyun, A.A. “Microbiological hazard analysis of ready-to-eat meats processed at a food plant in Trinidad, West Indies.” *Infection Ecology and Epidemiology* 3 (2013).

Türp, J.C., Spranger, H. “[Non-communicable disease and their significance for dental medicine].” *Swiss Dent J.* 126 (2016): 473-489.

Takahashi, H., Kimura, B., Tanaka, Y., Shinozaki, J., Suda, T., Fujii, T. “Real-time PCR and enrichment culture for sensitive detection and enumeration of Escherichia coli.” *J. Microbiol. Methods* 79 (2009): 124-127.

Tanaka, Y., Yamaguchi, N., Nasu, M. “Viability of Escherichia coli O157:H7 in natural river water determined by the use of flow cytometry.” *J. Appl. Microbiol.* 88 (2000): 228-236.

Taubes, -g. “The bacteria fight back.” *Science* 321 (2008): 356-361.

Taylor, E. “HACCP in small companies: benefit or burden?” *Food Control* 12 (2001): 217-222.

Taylor, E., Kane, K. “Reducing the burden of HACCP on SMEs.” *Food Control* 16 (2005): 883-839.

Taylor, L.H., Latham, S.M., Woolhouse, M.E. “Risk factors for human disease emergence.” *Philos Trans R Soc Lond B Biol Sci.* 356 (2001): 983–989.

Tayyem, R.F., Bawadi, H.A., Shehadah, I., AbuMweis, S.S., Agraib, L.M., Al-Jaberi, T., Al-Nusairr, M., Heath, D.D., Bani-Hani, K.E. “Meats, milk and fat consumption in colorectal cancer.” *J Hum Nutr Diet.* 14 (2016): [Epub ahead of print].

Te Morenga, L., Mallard, S., Mann, J. “Dietary sugars and body weight: systematic review and meta-analysis od randomised controlled trials and cohort studies.” *BMJ* 15 (2012): e7492.

Teuber, M. “Spread of antibiotic resistance with food-borne pathogens.” *Cell Molecular Life Science*, 1999: 755-763.

The Open University UK. “Hygiene and Environmental Health Module: 8. Food Contamination and Spoilage.” In *HEAT Higiene and Environmental Health, Part 1*, edited by http://www.open.edu/openlearnworks/mod/oucontent/view.php?id=187. 2016.

Thornton, P.K. “Livestock production: recent trends, future prospects.” *Philos Trans R Soc Lond B Biol Sci.* 365 (2010): 2853-2867.

Torgerson, P.R., Keller, K., Magnotta, M., Ragland, N. “The global burden of alveolar echinococcosis.” *PLoS Neglected Tropical Diseases* 4 (2010): e722.

Torgerson, P.R., Mastroiacovo, P. “The global burden of congenital toxoplasmosis: a systematic review.” *Bulletin if the World Health Organization.* 91 (July 2013): 501-508.

Tsen, H.Y., Chen, T.R. “Use of polymerase chain reaction for specific detection of type A, D and E enterotoxigenic Staphylococcus aureus in foods.” *Applied microbiology and biotechnology* 37 (1992): 685-690.

UN. *UN data.* 2012. http://data.un.org/ (accessed 07 05, 2016).

UNESCO. *United Nations Educationa, Scientific and Cultural Organization.* UNESCO. 2012. http://www.unesco.org/culture/ich/en/RL/mediterranean-diet-00884 (accessed 03 17, 2016).

USDA. *2015–2020 Dietary Guidelines for Americans.* 8th Edition. Washington: USDA Publication, 2015.

van Vliet, A.H.M., Ketley, J.M. “Pathogenesis of enteric Campylobacter infection.” *Journal of Applied Microbiology* 90 (2001): 45S-56S.

Vandamme, P., Debruyne, I., de Brandt, E., Falsen, E. “Reclassification of Bacteroides ureolyticus as Campylobacter ureolyticus comb. nov., and emmended description of the genus Campylobacter.” *Int J Environ Res Public Health* 10 (2010): 2016-2022.

Vasickova, P., Dvorska, L., Lorencova, A., Pavlik, I. “Viruses as a cause of foodborne diseases: a review of the literature.” *Vet. Med.* 3 (2005): 89-104.

Viegas, S., Cunha, I., Correia, C., Coelho, A., Maia, C., Pena, C., Bonito, C., Sousa, I., Toscano, MM., Furtado, R., Santos, S., Lopes, T., Saraiva, M. “Investigação interlaboratorial de toxinfecções alimentares, 2013.” *Observações_Boletim Epidemiológico* (INSA), no. 07 (2014): 03-06.

Violaris, Y., Bridges, O., Bridges, J. “Small businesses - big risks: current status and future direction of HACCP in Cyprus.” *Food Control* 19 (2008): 439-448.

Vlastarakos, P.V., Vassileiou, A., Delicha, E., Kikidis, D., Protopapas, D., Nikolopoulos, T.P. “Dietary consumption patterns and laryngeal cancer risk.” *Ear Nose Throat J.* 38 (2016): 32-38.

Walker, P. “Public health implications of meat production and consumption.” *Public Health Nutrition* 8 (2005): 348-356.

Wang, W.-J., Crompton, R.H. “The role of load-carrying in the evolution of modern body proportions.” *Journal of Anatomy* 204 (2004): 417-430.

Ward, T.J., Ducey, T.F., Usgaard, T., Dunn, K.A., Bielawski, J.P. “Multilocus genotyping assays for single nucleotide polymorphism-based subtyping of Listeria monocytogenes isolates.” *Applied environmental microbiology* 74 (2008): 7629-7642.

Wattiau, P., Boland, C., Bertrand, S. “Methodologies for Salmonella enterica subsp. enterica Subtyping: Gold Standards and Alternatives.” *Applied and Environmental Microbiology* 77 (2011): 7877-7885.

Wattiau, P., Van Hessche, M., Schlicker, C., Vander Veken, H., Imberechts, and H. “Comparison of classical serotyping and PremiTest assay for routine identification

of common Salmonella enterica serovars. J. Clin. Microbiol." *Journal of clinical microbiology* 46 (2008): 4037–4040.

Weese, J.S., Avery, B.P., Reid-Smith, R.J. "Detection and quantification of methicillin-resistant Staphylococcus aureus (MRSA) clones in retail meat products." *Letters in Applied Microbiology* 51 (2010): 338-342.

Weide-Btjes, M., Kobe, B., Lange, C., Schwarz, S. "Molecular typing of Salmonella enterica subsp. enterica serovar Hadar: evaluation and application of different typing methods." *Veterinary microbiology* 61 (1998): 215-227.

WHO. "Escherichia coli diarrhoea." *Bull WHO* 58 (1980): 23-36.

WHO. *Food and health in Europe: a new basis for action.* WHO Regional publications european series, 2004.

WHO. "Guideline: Sodium intake for adults and children." 2012.

WHO. "Guideline: Sugars intake for adults and children." 2015.

—. *Healthy diet.* 09 2015. http://www.who.int/mediacentre/factsheets/fs394/en/ (accessed 06 08, 2016).

—. *IARC Handbooks of Cancer Prevention, Weight control and physical activity.* Vol. 6. Lyon: IARC, 2002.

WHO. "Q&A on the carcinogenicity of the consumption of red meat andprocessed meat. ." World Health Organization. International Agency for Research on Cancer., 2015.

—. *World Health Organization.* 10 2015. http://www.who.int/features/qa/cancer-red-meat/en/ (accessed 03 19, 2016).

—. *World Health Organization.* 2011 йил October. http://www.who.int/mediacentre/factsheets/fs255/en/ (accessed 2015 йил 10-March).

Wilson, I.G., Cooper, J.E., Gilmour, A. “Detection of enterotoxigenic Staphylococcus aureus in dried skimmed mil: use of the polymerase chain reaction for amplification and detection of staphylococcal enterotoxin genes entB and entC1 and the thermonuclease gene nuc.” *Applied Environmental Microbiology* 57 (1991): 1793-1798.

Wooldridge, K.G., Ketley, J.M. “Campylobacter-host cell interactions.” *TRENDS in Microbiology* 5 (1997).

Wu, G. “Amino acids: Metabolism, functions, and nutrition.” *Amino Acids* 37 (2009): 1-17.

Wójcik, O. P., Koenig, K. L., Zeleniuch-Jacquotte, A., Costa, M., Chen, Y. “The potential protective effects of taurine on coronary heart disease.” *Atherosclerosis* 208 (2010): 19-25.

Xu, M., Huang, T., Lee, A.W., Qi, L., Cho, S. “Ready-to-Eat Cereal Consumption with Total and Cause-Specific Mortality: Prospective Analysis of 367,422 Individuals.” *J Am Coll Nutr* 23 (2015): 1-7.

Xu, S., Xue, Y. “Pediatric obesity: Causes, symptoms, prevention and treatment.” *Exp Ther Med.* 11 (2016): 15-20.

Yin, Y., Tan, W., Wang, G., Kong, S., Zhou, X., Zhao, D., Jia, Y., Pan, Z., Jiao, X. “Geographical and longitudinal analysis of Listeria monocytogenes genetic diversity reveals its correlation with virulence and unique evolution.” *Microbiological research* 175 (2015): 84-92.

Zhang, W., Jayarao, B.M., Knabel, S.J. “Multi-virulence-locus sequence typing of Listeria monocytogenes.” *Applied environmentla microbiology* 70 (2004): 913-920.

Zia, S., Wareing, D., Sutton, C., Bolton, E., Mitchell, D., Goodacre, J.A. *Health problems following Campylobacter jejuni enteritis in a Lancashire population.* Vol. 42. Rheumatology (Oxford), 2003.